

Major Histocompatibility Complex Class II Expression On Ovine T  
Cells.

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# ABSTRACT OF THESIS

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The expression of major histocompatibility complex (MHC) class II gene products is tightly regulated and is normally confined to professional antigen presenting cells (B cells, dendritic cells, monocytes). However many cells can be induced to express MHC class II molecules, for example human T cells do not express class II but synthesize and express high levels upon activation. In sheep, a percentage of resting T cells express class II and upon activation there is increased expression of the different MHC class II molecules (Dutia *et al*, 1993a). The significance of the increased class II expression after activation of T cells is unclear.

In this study the expression of MHC class II molecules on T cells from various immunological compartments of the sheep was examined. Monoclonal antibodies (mAbs) were characterized which react specifically with the homologues of human DR $\alpha$  and DQ $\alpha$  molecules. The expression of these antigens was established on B cells and T cell subsets derived from peripheral blood, efferent lymph and afferent lymph. B cells from each of the lymphocyte sources expressed both DR $\alpha$  and DQ $\alpha$  antigens. Variable expression on T cells was found. Each of the T cell subsets expressed both DR and DQ molecules at much higher levels in afferent lymph. DR expression on peripheral blood lymphocytes and efferent lymphocytes was higher than DQ on each of the T cell subsets. Afferent lymphocytes represent memory type cells and class II DR expression on these T cells may mark activation status.

Expression of the class II subtypes was also examined on *in vivo* activated T cells and correlated with expression of activation markers. Increased expression of class II molecules was coincident with the increased expression of activation markers in each of the T cell subsets with the exception of  $\gamma\delta$  cells. The lack of correlation on  $\gamma\delta$  T cells may be a reflection on the type of antigen used. Levels of DQ expression on CD4 positive cells after activation showed a more dramatic increase than levels of DR expression.

Cytokine profiles of concanavalin A (Con A) activated T cells (DR positive) were examined and compared with that of DR negative T cells. The data reveal that IL-6 mRNA production correlated with DR expression. IL-6 was induced after activation of the total T cell population and was not induced in the DR negative T cells. No detectable differences in IL-4, IL-10 and  $\gamma$ IFN mRNA profiles were observed between the total T cell population and the DR negative T cells.

The increased expression of class II molecules after *in vivo* activation coincided with the increased expression of activation markers. Correlation with activation markers did not provide further insight into the possibility that DR and DQ molecules may fulfill different functional roles whereas examination of cytokines secreted revealed that DR expression correlated with IL-6 production after Con A activation. This is indicative of a functionally distinct immunoregulatory role for DR positive T cells. The differential expression of class II molecules may fulfill distinct functions in regulating the outcome of T cell activation and the resultant effector function.

Using the MACS cell separation technique, a novel lymphocyte population was identified in sheep. These lymphocytes were CD3 positive suggesting they are of the T cell lineage, however they did not express markers for CD4, CD8 or  $\gamma\delta$  T cells. This null cell population represent approximately 5% of the efferent lymphocytes.

I hereby declare that the research described within this thesis is based entirely upon my own work.

Paula Keating.

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## ABBREVIATIONS

Ag	antigen
ATP	adenosine triphosphate
AP	ammonium persulphate
APC	antigen presenting cell
bp	base pair
BCG	bacille Calmette-Guerin
CD	cluster of differentiation
cDNA	DNA complementary to mRNA
CIIV	class II associated vesicle
CLIP	class II associated invariant chain peptide
ConA	concanavalin A
cpm	counts per minute
CsA	cyclosporin A
DEPC	dimethylsulphoxide
DNA	deoxy ribonucleic acid
EDTA	ethylenediamine tetra-acetate
ELISA	enzyme linked immunosorbent assay
ER	endoplasmic reticulum
FACS	fluorescent activated cell sorter
FSC	forward scatter
g	gravity
g, mg, µg	gramme, milligramme, microgramme
γIFN	γ Interferon
GM-CSF	granular macrophage colony stimulating factor
HBSS	Hanks buffered salt solution
HEV	high endothelial venules
HLA	human leukocyte antigen
HSA	human serum albumin
Hsa	heat stable antigen
Hsp	heat shock protein
HPEC	high performance electrophoresis chromatography
Ig	immunoglobulin
Ii	invariant chain
IL-2/3/4/5/6/10	Interleukin 2/3/4/5/6/10

kDa	kiloDalton
LMP	low molecular weight polypeptide
LPS	lipopolysaccharide
l, ml, $\mu$ l	litre, millilitre, microlitre
M, mM, $\mu$ M	molar, millimolar, micromolar
mAb	monoclonal antibody
MACS	Magnetic activated cell sorter
MBP	myelin basic protein
MHC	Major histocompatibility complex
MLR	mixed lymphocyte reaction
mRNA	messengerRNA
MS	multiple sclerosis
NF-ATp	nuclear factor of activated T cells
NK cell	Natural Killer cell
nm	nanometers
ov	ovine
Ova	ovalbumin
PAGE	polyacrylamide gel electrophoresis
PBL	peripheral blood lymphocytes
PBMC	peripheral blood mononuclear cells
PBS	phosphate buffered saline
PCR	polymerase chain reaction
PDA	piperazine diacrylamide
PE	phycoerythrin
PMSF	phenyl methyl sulphonyl flouride
PVDF	polyvinylidene difluoride
RNA	ribonucleic acid
RT-PCR	reverse transcriptase polymerase chain reaction
rpm	revolutions per minute
SA	streptavidin
SDS	sodium dodecyl sulphate
SSC	side scatter
SSC	sodium-salt-citrate
TAE	tris-acetate-EDTA
TAP	transporter associated with antigen processing
TCA	trichloroacetic acid
TCR	T cell receptor

TE	tris-EDTA
TEMED	tetramethylethylenediamine
TNF	Tumour necrosis factor
U	units
UV	ultraviolet
V, kV	Volts, kiloVolts
v/v	volume/volume
w/v	weight/volume

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## ABSTRACT

## DECLARATION

## ACKNOWLEDGEMENTS

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## CHAPTER 1 Introduction

The demonstration that peptide fragments bound directly to major histocompatibility complex (MHC) molecules (Babbitt *et al*,1985) provided the first evidence that MHC molecules interact with processed antigen independently of TCR engagement. Since then much has been learnt on antigen presentation by the MHC. The following review concentrates on the processing of antigen for presentation by MHC class II molecules and the functional outcome of antigen presentation.

### 1.1 The Major Histocompatibility Complex

#### 1.1.1 Genes and proteins of the MHC

Self/nonself discrimination was first demonstrated by the rejection of foreign tissue grafts in mice (Gorer,1936). The genetic loci involved in this rejection process were subsequently mapped to a region known as the major histocompatibility complex (reviewed in Klein,1986). The MHC is located on chromosome 17 in mice and on chromosome 6 in humans. Three classes of MHC molecules have been identified in the MHC complexes of both mouse and man, classes I, II and III (reviewed in Campbell and Trowsdale,1993; Trowsdale,1993). The class I and class II regions encode polymorphic cell surface molecules involved in immune recognition of antigens. The class III region encodes genes for the complement proteins C2, C4, and  $\beta$  factor, as well as genes for  $\text{TNF}\alpha$  and  $\beta$  and the heat shock protein (Hsp) 70. Other genes with no obvious association with the immune system have also been identified within the class III region.

In humans the class I loci contains around 20 genes, only three of which, the HLA-A, B, and C loci, produce polymorphic products that are clearly involved in antigen presentation. The other genes encoded in the HLA-E, F, G, H, I and J regions are characterized by limited polymorphism, restricted tissue distribution and low cell surface expression. The classical class I molecules associate with  $\beta 2$ -microglobulin in the presentation of endogenously derived proteins such as those from viruses. This allows the class I restricted CD8 T cells to screen for the expression of non-self material (reviewed in Monaco,1992).

The class II region in humans (HLA-D) is composed of three loci HLA-DP,

DQ and DR that are directly involved in antigen presentation. The HLA-DM molecules have also recently been found to play a critical role in antigen presentation (Morris *et al*,1994). Additional loci such as HLA-DN and DO are considered to be involved with antigen processing and transport to the endoplasmic reticulum (ER) (Ting and Baldwin,1993). TAP1 and TAP2 (for transporter associated with antigen processing) genes located in the class II region encode transporter proteins important for class I peptide translocation, while the closely associated LMP1 and LMP2 (for low molecular weight polypeptide) genes encode proteins similar to subunit proteins of proteasomes (Monaco and McDevitt,1982). Each of the DP, DQ and DR loci contain class II A and B genes which encode  $\alpha$  and  $\beta$  chains of the class II heterodimers. Class II molecules are usually associated with the presentation of exogenous proteins.

The major feature of the MHC is the polymorphism of some of the class I and class II genes. In general class I molecules are somewhat more polymorphic than class II molecules although some of the class I molecules exhibit little polymorphism.

## **1.2 The Class II region**

The human HLA-DP and DQ subregions contain pairs of polymorphic A and B genes. In the DP region both members of a DPA/DPB gene pair are pseudogenes. Within the DR region a single non-polymorphic  $\alpha$  chain can be associated with the product of several DRB genes, the number of which varies between haplotypes (Bodmer *et al*,1990). In mouse the functional class II genes are grouped into two subregions (IA, IE) which code for E $\alpha$ E $\beta$  and A $\alpha$ A $\beta$  class II molecules. Genes homologous to the HLA-DN have also been identified (Karlsson and Peterson,1992). In the IO region the O $\beta$  protein product has been identified (Karlsson *et al*,1991) and the IOA gene found to be homologous to HLA-DN (Karlsson and Peterson,1992).

*In situ* hybridization has localized the MHC gene region of sheep (*Ovar-D*) to chromosome 20 (Mahdy *et al*,1989). Genes equivalent to the HLA-DR, DQ and DN homologues have been identified. No DPA genes were identified (Scott *et al*,1987; Deverson *et al*,1991). Using two unrelated sheep, 7 A and 24 B genes have been detected using human/murine cDNA probes (Deverson *et al*,1991). The A genes were identified as DR-like, DQ-like and DN-like, the B genes could not be determined because of extensive cross-hybridization. From these cloned genes a

DRA and a DRB gene have been identified and shown to be expressed on transfection into mouse L cells (Ballingall *et al*,1992). Two DQA genes, a DQB gene and a DRB pseudogene have also been sequenced (Scott *et al*,1991a&b). Alleles within the DRA, DQA1, DQA2 and DQB regions have been identified (Fabb *et al*,1993; van Oorschot *et al*,1994). These regions in sheep are thus polymorphic. The protein products of DR and DQ molecules have been identified using mAbs (Puri and Brandon,1987; Dutia *et al*,1994). One DQ $\alpha$ , DR $\alpha$ , DQ $\beta$  and two DR $\beta$  molecules have so far been found to be expressed (Ballingall *et al*,1992; Dutia *et al*,1993&4). A distinguishing feature of the ovine class II region which differs from the HLA class II region is the presence of a DY region (Wright *et al*,1994). The DY region is represented by an A/B gene pair.

### 1.2.1 Gene regulation

Class II molecules are expressed primarily on B cells, thymic epithelia, macrophages and dendritic cells (reviewed in Kappes and Stominger,1988; Benoist and Mathis,1990; Glimcher and Kara,1992). The expression of class II is under tight developmental control on these cells. For example, class II expression is absent on murine pre-B cells but appears in mature B cells, the differentiation of B cells into plasma cells is accompanied by a loss in class II expression. Ovine B cells have also been shown to lose expression of class II on terminal differentiation into plasma cells (Dutia *et al*,1993a). Expression of class II is also subject to regulation by cytokines. Macrophages, endothelial cells and astrocytes can be induced to express class II by  $\gamma$ IFN (Wong *et al*,1984; Manyak *et al*,1988; Celada and Maki,1991).

Expression is controlled at the level of transcription, and specific cis-acting sequences have been recognized in the proximal promoters of the MHC class II genes. These are the highly conserved X, Y and W/Z boxes. Each of these promoter elements appear to be involved in the regulation of both constitutive and inducible class II gene expression (reviewed in Glimcher and Kara, 1992). An additional promoter element has been identified on the DRA gene. The DRA gene contains an octamer motif which appears to be involved in B-cell specific gene expression as mutation of this element reduces the expression of DR $\alpha$  (Tsang *et al*, 1990). Upstream J elements have been found in HLA-DPA and HLA-DQB promoters that are necessary for constitutive and  $\gamma$ IFN induced gene expression (reviewed in Ting and Baldwin,1993). A number of nuclear binding proteins have been identified that

bind to these class II X, Y or W/Z box sequences with varying affinities for the different class II box sequences (Ono *et al*,1991; Ting and Baldwin,1993). *In vivo* analysis of class II promoters has demonstrated that the regulated expression of class II genes is accompanied by changes in promoter occupancy implying that promoter accessibility is likely to be the major determinant of class II expression. Thus changes in transcription of class II genes are associated with changes in factor binding at the promoter *in vivo* (Kara and Glimcher,1993).

Bare lymphocyte syndrome (BLS) is a rare genetic disease which causes severe immune combined immunodeficiency due to a complete lack of expression of class II molecules. Using B cell lines established from BLS patients two types of HLA class II regulatory mutants have been distinguished at the molecular level. One type is characterized by the lack of binding by factor RF-X to the X box on the HLA promoter (Reith *et al*,1988). Unoccupied promoters have also been identified *in vivo* (Steimle *et al*,1993). In the second type of BLS no defects in promoter occupancy have been identified and the defect is due to the absence of CIITA transactivator gene expression (Steimle *et al*,1994). The defect in these cell lines is fully corrected by the CIITA gene. Control of both constitutive and inducible class II expression is also mediated by the transactivator CIITA gene. Steimle and co-workers showed that CIITA expression is induced by  $\gamma$ IFN. Furthermore, a functional CIITA gene is sufficient to activate expression of class II genes in class II negative cells in the absence of  $\gamma$ IFN. Thus CIITA acts as a general regulator of both inducible and constitutive class II expression. As there is no evidence of CIITA binding directly to a class II promoter, it was suggested that CIITA may act as a tissue specific co-activator (Steimle *et al*,1994).

### **1.2.2 Structure of MHC class II molecules**

Early structural studies showed that the human class II molecules are  $\alpha\beta$  heterodimers in which both chains span the cell membrane (Springer *et al*,1977). The heterodimer is composed of the  $\alpha$  chain non-covalently linked to the  $\beta$  chain. The  $\alpha$  subunits have an approximate molecular weight of 33-35kD and the  $\beta$  subunits of 25-30kD. The extracellular region is divided into two domains of 90-100 amino acids each, a transmembrane region of 20-25 amino acids and a short intracellular region of 12-15 amino acids. The membrane proximal domains ( $\alpha 2$  and  $\beta 2$ ) each contain an internal disulphide bond and are structurally very similar to immunoglobulin domains. The membrane distal domains ( $\alpha 1$  and  $\beta 1$ ) have no



such homology and associate to form a structure that constitutes the peptide binding region of the molecule (reviewed in Kappes and Strominger,1988; Cresswell,1994). From the x-ray crystallographic analysis of class II (Brown *et al*,1993) the peptide binding structure consists of eight strands of anti-parallel  $\beta$  sheets with 2 anti-parallel  $\alpha$  helical regions overlaying them. There is a deep cleft between the  $\alpha$  helices which accommodates the peptide. In addition, the majority of allelic polymorphisms in both mouse and human class II molecules are located within the  $\alpha 1$  or  $\beta 1$  domains (Bell *et al*,1985). Similar MHC class II proteins have been described in sheep. As in mice and humans, sheep  $\alpha$  and  $\beta$  chains are glycosylated with complex N-linked oligosaccharides (Puri *et al*,1987a ;Dutia *et al*,1990a).

### 1.2.3 Biological expression of MHC class II gene products

Class II expression occurs constitutively on classical antigen presenting cells (APCs) such as Langerhan's cells of the skin, dendritic cells, macrophages, monocytes and B cells in all species so far examined (Kappes and Strominger,1988). In humans, specific epithelial cells (skin keratinocytes, intestinal epithelium, thymic epithelium, kidney tubular cells, vascular epithelium), astrocytes and melanocytes transiently express class II molecules. The level of class II expression is not static in these cells but can be up-regulated or down-regulated by a large number of external stimuli. The major regulator of class II expression is  $\gamma$ IFN.  $\text{TNF}\alpha$  is also an inducer of class II gene expression and in macrophages and monocytes it acts synergistically with  $\gamma$ IFN. In a variety of cell lines derived from malignant solid tumours, class II expression is inducible with  $\gamma$ IFN or mitogens (reviewed in Benoist and Mathis,1990; Glimcher and Kara,1992).

Striking differences between species in the expression of MHC class II on T cells are described in the literature. The synthesis of class II molecules has been demonstrated directly on human T cells (Oshima and Eckels,1990a), rat T cells (Reizis *et al*,1994; Broeren *et al*,1995) and sheep T cells (Hopkins *et al*,1993). In human and rat T cells class II expression only occurs following activation, while in the sheep a proportion of T cells are class II positive and show increased expression following activation. All equine (Crepaldi *et al*,1986), feline (Rideout *et al*,1992) and canine (Doveren *et al*,1986) T cells express class II molecules constitutively. In the cat increased expression of class II has been demonstrated on



T cells following viral infections (Rideout *et al*,1992).

In contrast, the expression of class II molecules on mouse T cells is controversial. Several groups have reported the phenotypic expression of MHC class II determinants on mouse T cells both *in vitro* (Singh *et al*,1984; Bevan and Chisholm,1986) and *in vivo* (Gautam *et al*,1991). However others have demonstrated that class II molecules on mouse T cells may be passively adsorbed from antigen-presenting stimulator cells (Lorber *et al*,1982). Reizis *et al* (1994), recently reported that a significant amount of class II proteins on the surface of activated rat T cells is acquired by passive adsorption, nonetheless active synthesis was found to take place in the same cells. Thus active expression and passive acquisition of class II proteins may occur simultaneously in murine T cells.

On mature human B cells there is co-ordinate expression of DP, DQ and DR loci (Nunez *et al*,1984). On human T cells the products of DR, DP and DQ loci are not co-ordinately expressed and there are differences between CD4 and CD8 T cell populations in levels of expression (Diedrichs and Schendel,1989; Oshima and Eckels,1990a). Similarly in sheep there is differential expression of class II molecules on T cell subsets. In foetal sheep blood there is no expression of class II on either CD8 or  $\gamma\delta$  T cells with a very low level of expression on CD4 T cells. In adult peripheral blood and efferent lymph a significant proportion of T cells express DR but not DQ while in afferent lymph all three T cell subsets express both DQ and DR (Dutia *et al*,1993a).

### **1.3 MHC Polymorphism**

Diversity in MHC expression affects immunological potential in several ways. Polymorphisms in MHC molecules influence the ability of MHC to bind a given peptide. Consequently individuals expressing different MHC alleles select different peptides of a given antigen for presentation. Thus MHC polymorphism influences the spectrum of peptide antigens against which an individual can initiate an immune response. There is also an association of MHC phenotypes with susceptibility to certain diseases. This may be a consequence of the selective nature of the binding groove ensuring certain alleles are capable of binding pathogenic peptides. For example in coeliac disease the antigen gliadin has a strong association with HLA-DQ\*0501 and HLA-DQ\*0201. Gliadin reactive T cells have been shown to be restricted to these two alleles (reviewed in George *et al*,1995). Other

diseases associated with HLA alleles include insulin dependent diabetes mellitus and pemphigus vulgaris. With these diseases it has been demonstrated that it is polymorphic variations in MHC molecules themselves, rather than hypothetical disease genes closely linked to MHC loci that contribute to the disease propensity. Autoimmune disease (eg. multiple sclerosis, rheumatoid arthritis, pemphigus vulgaris, insulin dependent diabetes mellitus) associations implicate HLA-DQ more frequently than DR alleles in predisposition to disease (Altmann *et al*,1991). Residues critical for peptide binding have been associated with susceptibility to autoimmune diseases (Hammer *et al*,1995). Two polymorphic positions in the MHC class II  $\beta$  chain are critical for selective peptide binding and the development of autoimmunity (position 71 of DR $\beta$  and position 57 of DQ $\beta$ ). In autoimmune diseases associated with DR4 alleles, the charge at position DR $\beta$ 71 controls the charge at peptide position P4 (relative to the first anchor P1). In rheumatoid arthritis (RA) patients, RA peptides have a negative charge at P4 whereas pemphigus vulgaris peptides have a positive charge at the same position (Wucherpfennig and Strominger,1995). It appears that polymorphic residues critical in peptide binding are associated with susceptibility to the development of autoimmune disease.

#### **1.4 MHC class II assembly and antigen presentation by antigen presenting cells (APCs)**

##### **1.4.1 Assembly and transport of Class II, the role of invariant chain**

MHC class II molecules are assembled in the endoplasmic reticulum (ER) (Kvist *et al*,1988) and only the functional unit of the  $\alpha\beta$  heterodimer leaves the ER (Sant *et al*,1991). During biosynthesis of class II a third chain, the invariant chain (Ii) associates transiently with the class II  $\alpha\beta$  chains. The invariant chain was so named because of its lack of allelic polymorphism between different strains of mice. The Ii is not MHC linked. Ii rapidly forms noncovalently associated trimers in the ER (Marks *et al*,1990) and class II  $\alpha\beta$  dimers associate with these trimers to form nonamers (Roche *et al*,1991). In the human there are 4 primary forms of Ii, called p33, p35, p43 and p45. The N-terminal cytoplasmic extension found on human Ii p35 and p43 forms, constitute a strong ER retention signal (Bakke and Dobberstein,1990). It is thought that the formation of mixed trimers of Ii which will include either p35 or p43 will exert their retention signal and as a consequence all non-class II associated Ii is retained within the ER (Cresswell,1994). Upon association of the class II  $\alpha$  and  $\beta$  subunits with invariant

chain the ER retention signal is negated (Machamer and Cresswell,1982). How this assembly inhibits li chain mediated retention is unknown.

Association with li is not an absolute requirement for assembly and surface expression of the  $\alpha\beta$  heterodimer. Using cell lines expressing class II  $\alpha\beta$  subunits but not li, class II expression on the cell surface was demonstrated (Sekaly *et al*,1986). Later experiments have revealed that li facilitates assembly and transport of the  $\alpha\beta$  heterodimer (Anderson and Miller,1992). Evidence from li knockout mice further supports an important functional role for li in class II assembly and transport. Invariant chain knockout mice have impaired class II expression, dramatically reduced numbers of CD4<sup>+</sup> T cells and spleen cells that are defective in their ability to present antigens to T lymphocytes (Bikoff *et al*,1993). Furthermore li knockout mice have an unstable class II configuration affecting processing and presentation of native protein antigens, whereas peptides are presented very efficiently (Acolla *et al*,1995). Thus it would appear that li does in fact facilitate antigen processing.

After assembly of the  $\alpha\beta$ li complexes, they move out of the ER and through the Golgi stacks. When they reach the trans-Golgi they deviate from the typical exocytic pathway and move to an endocytic pathway (Neefjes *et al*,1990). The li is thought to drive class II molecules into the endosomal system. It has been demonstrated that li augments class II surface expression by improving class II egress from the ER (Layet and Germain,1991). Lotteau *et al* (1990), identified a targetting signal on the cytoplasmic tail of li which directs traffic to the endosomes.

li association also prevents the association of peptides with class II molecules before they are delivered into the endosomal system (Cresswell,1994). As the accepted function of class II molecules is presentation of exogenous antigen to T cells the association of li with the dimer in the ER prevents the binding of endogenous peptides. The li possesses a short internal segment (the class II associated invariant chain peptide, CLIP) that interacts with the binding groove such that binding cannot occur (Germain,1994). Polymorphic differences may affect the assembly of li with allelic variants of class II (Bikoff *et al*,1995). In the absence of li the A $\alpha^b$ A $\beta^b$  haplotype chains were expressed as free A $\alpha^b$  and A $\beta^b$  chains, however other allelic products assembled efficiently and were conformationally similar to wild type heterodimers.

Thus the Ii fulfills three important functions in the biosynthesis of class II molecules, it guides the folding of class II molecules, it targets delivery of class II to the endocytic route and it prevents premature binding of antigenic peptide to class II molecules.

#### 1.4.2 The endosomal route

After leaving the ER the assembled  $\alpha\beta$ Ii complex traverses the Golgi. However unlike most membrane glycoproteins, the class II-Ii complex is not immediately expressed on the cell surface. The pathway involved is unclear but early endosomes, late endosomes and lysosomes have all been implicated in various cell types. Precisely where in the endosomal/lysosomal system peptides bind and the mechanisms which regulate the process are unknown.

It was originally the work of Jensen (1990) that showed enhanced peptide binding to purified class II molecules in solution at acidic pH. At acidic pH the Ii chain derived CLIP peptide dissociates more rapidly (Riberdy *et al*, 1992). Mellins *et al* (1990), studying a cell line lacking the capacity to present intact antigens to T cells documented that its class II molecules dissociated into  $\alpha$  and  $\beta$  monomers on SDS gel electrophoresis. The association between failure to present antigen and loss of MHC class II dimer stability suggested that peptides were important for stable dimer state. From these observations it has been proposed that at neutral pH, the class II molecule cannot undergo the conformational change needed to attain the antigen bound state, even in the presence of peptide. At lower pH, the class II molecule is more flexible and receptive to antigen. When neutralization occurs class II molecules with bound peptide are locked in this state trapping the associated peptide (Sadegh-Nasseri and Germain, 1992). The class II DM molecule has also been shown to play an essential role in antigen presentation (Morris *et al*, 1994). DM is thought to maintain peptide free class II molecules by keeping the class II binding pocket free of CLIP (Stebbins *et al*, 1995).

In APCs a different population of vesicles distinct from endosomes or lysosomes have been identified (Amigorena *et al*, 1994). These class II associated vesicles (CIIV) are a specialized subset of the endosomal compartment. Morphological studies on CIIV in B cells reveal spherical structures with abundant internal membrane vesicles or infoldings (Amigorena *et al*, 1994). Schmid and Jackson (1994), have proposed the following route of intracellular antigen

presentation in APCs. Newly synthesized  $\alpha\beta$ li complexes leave the Golgi and enter early endosomal compartments. Degradation of the li occurs in this acidic and mildly proteolytic environment. Class II complexes are diverted to the CIIV where most of li is degraded and class II molecules are receptive for antigenic peptide. The CIIV mature into a high density compartment in which peptide loaded class II molecules accumulate. Receptor bound antigens are targeted to the CIIV from early endosomes to generate a diverse array of antigenic peptides. When peptides are loaded onto class II molecules, the aggregated complexes dissociate and the peptide-class II complex is transported to the cell surface for presentation to T cells. Recent evidence from Amigorena *et al* (1995) supports this theory. It was found that the CIIV hosts the final steps in the dissociation of li from class II molecules and the loading of antigen derived peptides into newly synthesized  $\alpha\beta$  dimers.

Through the conventional endosomal pathway presumably any cell can present different antigens to T cells. The CIIV in APCs may provide a particularly efficient means of presenting antigen. Much has yet to be learnt of the transport molecules, chaperones and carrier proteins involved in the route of class II movement from the site of loading to the cell surface.

#### **1.4.3 Antigen processing and presentation**

While the antigens presented by class II molecules are generally characterized as exogenous, it is required that they be internalized for processing in the endosomal route. This was demonstrated in the studies of Gell and Benacerraf (1959). They were the first to show that whilst B cells primarily recognize determinants found on native molecules, T cells can recognize both native and denatured forms of the antigen. It has since been established that antigens require some form of processing prior to presentation to T cells. This processing involves internalization of antigen and reappearance on the cell surface in association with class II for recognition by T cells.

Professional APC utilize different strategies for antigen capture. B cells present their antigen to T cells in a very efficient manner. Antigen specific immunoglobulin molecules on the surface of B cells can serve as a means for the cell to specifically take up and concentrate the antigen. This antigen can then be processed and re-expressed on the surface of B cells in association with class II. Because of their specificity and concentrating effect B cell presentation can occur at



concentrations much lower than those required by macrophages and dendritic cells (reviewed in Lanzavecchia,1990). In comparison to B cells, macrophages and dendritic cells acquire antigen in a non-specific manner. Macrophages are highly phagocytic and are likely to ingest antigen spontaneously. Fc receptors on macrophages enhance processing by binding and internalizing antigen-antibody complexes while complement receptors on macrophages help in the phagocytosis of complement opsinized cells (reviewed in Unanue and Allen,1987). The dendritic cell is a much more potent stimulator of T cells and has the unique capacity to induce primary immune responses. Dendritic cells appear to exist in an immature stage in non-lymphoid organs (e.g. Langerhans cells in the skin) where they ingest antigen. Dendritic cells utilize mannose receptors, Fc receptors and pinocytosis to capture antigen. After antigen uptake dendritic cells move to T cell areas of lymphoid organs where they mature into antigen presenting cells. Increased expression of adhesion molecules and presentational molecules (class II and CD1) allow them the potential to interact with both  $\alpha\beta$  and  $\gamma\delta$  T cell subsets (reviewed in Bujdoso *et al*,1990).

After binding to receptors on the plasma membrane, ligand-receptor complexes cluster in clathrin coated pits that subsequently invaginate, pinch off the membrane and form vesicles. The vesicles are then delivered to the endosomal route. Enhancing the rate of endocytosis of protein antigens by APC has been found to increase the efficiency with which they are processed for class II T cell recognition. The surface immunoglobulin of B cells can increase the efficiency of antigen presentation 3 fold (Lanzavecchia,1985). Fc receptors expressed on APC may also enhance processing by binding and internalizing antigen-antibody complexes (Cresswell,1994).

A fraction of naturally occurring class II associated peptides originate from cytoplasmic proteins. Cytosolic proteins may enter lysosomes through a process of autophagy, the engulfment of cytosolic material or even organelles (Dunn,1990). Following fusion of autophagocytic vesicles with lysosomal elements, cytosolic proteins are broken down and presented by class II molecules. Another route proposed for the presentation of cytosolic proteins is transport via heat shock proteins. Molecules of the Hsp 70 family have been implicated in a shuttle mechanism for the provision of processed antigen to class II in endocytic compartments (DeNagel and Pierce,1991). Hsp molecules bind peptide or degraded protein and thus prevent their complete degradation in the proteolytic environment

of lysosomes. Thus peptide from endogenous and exogenous antigen may be generated at the same location and available for binding to class II. This fits with studies on the purified peptides from class II molecules which reveal that almost all are endocytically acquired (Hunt *et al*,1992; Chicz *et al*,1993).

The length of the peptide presented to the T cell varies from 13-25 amino acids. The x-ray crystal structure of human HLA-DR1 (Brown *et al*,1993) revealed that the class II binding groove is open at both ends with an overall linear contact length of 15 residues. This differs from the class I peptide binding groove which is blocked at either end. Severe restrictions are imposed on the size of peptide class I can accommodate (8-10 residues), longer peptides are forced to bulge out in the middle (Guo *et al*,1992). The class II binding site allows peptide to protrude from it and consequently longer peptides can bind. The structure of the complex of DR1 and the antigenic peptide of influenza hemagglutinin shows that the peptide binds as a straight extended chain with a pronounced twist (Stern *et al*,1994). This confirmation allows most of the peptide residues to participate in the surface presented to the T cell receptor. Peptide residues at positions P4, P6, P7 and P9 (relative to the first anchor P1) appear to contribute to the specificity of peptide binding to different DR molecules (Stern *et al*,1994). Twelve of the hydrogen bonds involve residues conserved in most human and mouse class II alleles. This suggests a universal method for peptide binding by class II proteins. Several promiscuous peptides, capable of binding to different class II alleles, have been identified (Sinigaglia *et al*,1988; Panina-Bordignon *et al*,1989)

Class II molecules make several contacts with the peptide side chains to increase the overall binding affinity of the bound peptides. Side chain contacts differ between different alleles and determine the peptide sequence specificity for each class II allele (Sinigaglia and Hammer,1995). *In vitro* studies demonstrate that peptide-class II complexes have a long half-life (22hours). This is dependent on the length and sequence of the bound peptide. The half-life is not decreased by the presence of an excess concentration of other peptides which have greater affinity. Peptides which stabilized class II molecules in the presence of SDS conferred longer half-lives (Nelson *et al*,1994).

## **1.5 The functional outcome of antigen presentation by MHC class II to T cells**

### **1.5.1 T cell activation**

Once peptide-MHC complexes have been exported to the cell surface they function by interacting with the clonally distributed receptors of T lymphocytes (TCR). The process of lymphocyte activation is highly complex and involves integration of many signals that together determine the outcome. In addition to the TCR several co-receptors on the APC are involved. Some of these interactions function to strengthen the adhesion of the APC and the T cell while others provide important costimulatory signals. As a consequence of signal transduction events that follow stimulation, T cells produce a variety of cytokines. The cytokines produced regulate the subsequent development of the immune response by influencing proliferation, differentiation, migration and function of immune effector cells.

### **1.5.2 The ligand for peptide-MHC class II complex**

T cells recognize antigen through their T cell receptors in the form of peptide fragments bound to MHC. Each TCR has a binding affinity directed to a specific peptide-class II complex. This unique binding affinity arises from the structure of the combined variable domains within the  $\alpha$  and  $\beta$  chains making up individual TCR molecules. Mature T cells with  $\alpha\beta$  TCR come in two major classes, expressing either CD4 or CD8. CD4 T cells recognize peptide fragments bound to class II molecules while CD8 T cells recognize peptide fragments bound to class I molecules (Swain,1983). MHC class I and class II act as restriction elements in the antigen specific activation of CD8 and CD4 T cells respectively. The TCR and CD4/CD8 molecules diffuse independently on the plane of the T cell membrane and are brought together by co-recognition of the same peptide-MHC complex (reviewed in Springer,1990). The CD4 molecule interacts with the membrane proximal regions of class II that are relatively invariant while the TCR binds the polymorphic peptide binding region (reviewed in Janeway,1992). A measure of affinity of TCR for peptide-MHC complexes has come from competition experiments using soluble MHC complexed with specific peptides. This affinity with a  $K_d$  of  $5 \times 10^{-5} M$  is several orders of magnitude lower than that of most antibodies for their protein antigens suggesting that co-stimulation events are important in T cell



activation (Jorgensen *et al*,1992).

### 1.5.3 Peptide antagonists

Recent observations on T cell responses following TCR interaction with altered peptide ligands have highlighted the complexity of TCR signalling. Using altered peptide ligands which bind to defined MHC class II molecules, peptides have been identified which bind to the TCR and block the induction of normal signals. The variant peptides act as antagonists blocking positive responses to the wild type peptide presented by competent APCs (DeMagistris *et al*,1992). T cell anergy can be induced by variant peptides even in the presence of effective co-stimulation. In addition to peptide variants blocking the T cell response to immunogenic peptide, peptide variants also block the T cell response to superantigens. As superantigens bind at a different site to peptide, this effect appears to occur through the TCR. This finding also eliminates the possibility that TCR antagonism is the result of competition between peptides for binding sites (Evavold *et al*,1993). Thus the TCR appears to be able to alter effector function selectively. Conformational changes in the receptor itself may play a critical role in TCR signalling (Janeway,1995).

### 1.5.4 Signalling through the TCR

The TCR is found on the surface of all T cells. It is a complex of oligomeric structures composed of 6/7 different subunits. These are divided into three distinct groups of proteins, the receptor chains, the CD3 chains and the  $\zeta$  chains. The immunoglobulin-like clonotypic chains responsible for ligand binding are present as heterodimers. There are two forms of receptor:  $\alpha\beta$  and  $\gamma\delta$ . In sheep,  $\alpha\beta$  T cells are found on most mature T cells while  $\gamma\delta$  T cells constitute a major T cell population in sheep 3-4 months old (Mackay,1991). The CD3 chains encompass 3 distinct subunits  $\gamma$ ,  $\delta$  and  $\epsilon$ . The final group is the  $\zeta$  dimers, three proteins comprise the  $\zeta$  family. Of all these proteins the CD3 $\epsilon$  has been identified as having signalling capabilities (LeTourneur and Klausner,1992) while the cytoplasmic tail of  $\zeta$  chain appears to be essential for antigen stimulated signalling (Frank *et al*,1990).

CD4 and CD8 molecules are also important in signalling. The first indication that CD4 and CD8 play an active role in TCR mediated cell signalling came from studies demonstrating that anti-CD4 and anti-CD8 mAbs inhibited mitogen-induced activation in the absence of MHC ligands (Janeway,1988). The cytoplasmic

domains of both CD4 and CD8 bind the tyrosine kinase p56lck (Turner *et al*, 1990) leading to tyrosine phosphorylation of the  $\zeta$  chain of the TCR unit (Veillette *et al*, 1989). The proportion of cellular lck associated with CD4 is 10-20 fold higher than associated with CD8. Furthermore, aggregation of CD4 results in a more than 10-fold induction in the kinase activity of cellular lck, while aggregation of CD8 has barely detectable effects. It appears that CD4 and CD8 have distinct roles in regulating antigen receptor signalling during T cell activation (reviewed in Julius *et al*, 1993). Antigen presented by class II is brought into proximity with the TCR through binding the same ligand and the interaction of CD4 with TCR appears to be critical for lck activation (Janeway, 1992). The potentiation of T cell activation by CD4/CD8 is manifest as a 30-300 fold reduction in ligand density required for T cell activation.

Stimulation through the TCR activates the tyrosine kinases p59fyn, p56lck and ZAP-70 (Chan *et al*, 1992). Subsequently phospholipaseC- $\gamma$ 1 is activated by tyrosine phosphorylation, it cleaves phosphatidyl-inositol bisphosphate to the two active second messengers inositol 1,4,5-trisphosphate and diacylglycerol. These second messengers induce a sustained rise in intracellular calcium and activation of protein kinase C. The increased levels of calcium and the activation of protein kinase C in turn synergize to activate transactivating factors required for cytokine gene transcription (reviewed in Schwartz, 1992).

The transcription of cytokine genes in activated CD4 T cells is blocked by the immunosuppressive drug cyclosporin A (CsA). Calcineurin, one of the enzymes activated following increased intracellular calcium is a primary target of CsA (Schreiber and Crabtree, 1992). This suggests that calcineurin plays an essential role in regulating transcription of cytokines. NF-ATp (nuclear factor of activated T cells) is a substrate for calcineurin and it appears to be involved in the regulation and induction of several cytokine genes (Rao, 1994). Other nuclear complexes known to regulate cytokine gene expression include NF- $\kappa$ B, AP-1, AP-3 and octamer binding factors (Fraser *et al*, 1993).

The crystallization of the HLA-DR molecule revealed that the molecule crystallized as a dimer (a dimer of the heterodimer) such that the two molecules are parallel and orientated in the same direction (Brown *et al*, 1993). Dimers of heterodimers have also been observed in immunoprecipitates of class II molecules (Schafer and Pierce, 1994). There has been much speculation on the role of

superdimers in TCR signalling. One hypothesis is that the close approximation of two TCRs brought about by simultaneous recognition of two peptide-MHC ligands in each superdimer may modulate the TCR response. At increasing ligand density, there is an increased possibility that both molecules of the dimer are peptide associated and by physical proximity of all participating subunits a strong TCR signal would be elicited. At lower ligand density crosslinking of the two receptors may occur but only one may bind activating peptide and thus a reduced activation signal elicited. Thus through MHC class II dimer presentation of antigen the TCR may signal CD4 T cells in different ways (Janeway and Bottomly, 1994).

The interaction of TCR with MHC-peptide complex appears to be one of low affinity and short duration. This phenomenon has recently been explained by the finding that a small number of peptide-MHC complexes can serially trigger a much larger number of TCRs leading to an amplified and sustained signal. The high off-rate of TCR allows a single MHC-peptide complex to engage many TCRs. One MHC-peptide complex can trigger up to 200 TCRs. This is correlated with down-regulation of the TCRs that were specifically triggered, the bystander TCRs being essentially unaffected (Valitutti *et al*, 1995). The long half-life of peptide associated class II favors the potentiation of increased TCR signalling.

#### **1.5.5 Signalling through the APC-MHC class II**

In addition to their function in presentation of antigen to CD4 T cells, class II molecules transduce signals which regulate APC cell function. The capacity of MHC class II molecules to deliver signals that modulate APC cell function has been demonstrated with studies using anti-class II mAbs and superantigens. Superantigens bind with high affinity to MHC class II molecules and act as superantigens by bridging TCR molecules on T cells and class II molecules on APCs. Using these methods to crosslink class II molecules sustained LFA-1 adhesion on human B cells has been reported (Fuleihan *et al*, 1991). In addition, engagement of class II on B cells induces proliferation and immunoglobulin production (Mourad *et al*, 1989). On monocytes induced secretion of IL-1 has been reported (Palacios, 1985). Furthermore during T-B cell interaction, engagement of the TCR with class II - antigen complex delivers a signal to the B cell which requires the cytoplasmic domain of the class II molecule and results in surface CD80 (B7) expression (Nabavi *et al*, 1992). The CD40 ligand may be another molecule on activated T cells which induces CD80 (B7) expression by B cells (Ranheim and

Kipps,1993). Signal transduction through class II on B cells leads to two distinct signal transduction phenotypes depending on the B cells activation status. Crosslinking class II on non-primed murine B cells leads to rapid increases in cAMP levels and translocation of protein kinase C to a detergent insoluble fraction. These events mediate the induction of CD80 expression. Crosslinking class II on primed B cells leads to protein tyrosine kinase activation and calcium mobilization (Cambier *et al*,1991). The activation of protein tyrosine kinase triggers a wave of secondary activation events for MHC class II mediated effector functions such as adhesion and induction of cytokines. Both signalling pathways play important roles in T-B cell collaboration (reviewed in Wade *et al*,1993; Scholl and Geha,1994).

In addition to T cell receptor interaction with MHC class II antigen complexes, a successful outcome of T-B cell collaboration requires participation of additional transmembrane receptor ligand pairs. Some of these are constitutively expressed while others like CD80 are induced by class II/TCR interactions.

#### **1.5.6 Co-receptors**

Adhesion molecules enhance signal transduction between the T cell and the APC by increasing occupancy of the TCR. CD2, LFA-1 (CD11a\CD18) and their ligands LFA-3 (CD58) and ICAM-1 (CD54) respectively, are the major adhesion molecules that contribute to the T cell- APC binding avidity. Engagement of the TCR by mAbs has been shown to upregulate the affinity of LFA-1 for its counter receptor (Dustin and Springer,1989). Class II molecules can similarly transduce intracellular signals that activate LFA-1 molecules (Mourad *et al*,1990). Enhanced expression of CD2, LFA-3 and LFA-1 has also been reported on activated T cells (Sanders *et al*,1988; Mackay *et al*,1990). Increasing cellular adhesion between APC and T cell augments the T cell response.

Two costimulatory molecules with a unique role in T cell activation have been identified. These are B7 (CD80) and heat stable antigen (Hsa). They provide co-stimulatory signals which are essential for T cell proliferation and differentiation but do not by themselves induce any response in T cells. However when the T cell has its receptor ligated and engages the B7 and Hsa ligands the T cell will proliferate and differentiate. T cells which do not receive co-stimulatory signals are thought to die (Webb *et al*,1990) or to become anergic (Jenkins *et al*,1988). Thus an encounter with antigen can lead to two distinct outcomes which

are dependent on costimulatory signals.

CD80 was first identified on activated B cells, and expression was shown to be upregulated during B cell activation (Freeman *et al*,1989). It has since been documented that CD80 expression is not limited to activated B cells but is more generally characteristic of APCs (Linsley and Ledbetter,1993). Crosslinking with CD28 induces rapid tyrosine phosphorylation of proteins and these signals appear to be distinct from those delivered via the TCR. CD80 has also been identified as a ligand for CTLA-4 receptors found only on antigen experienced T cells (Linsley *et al*,1991b), which bind CD80 with a 20 fold higher affinity than CD28 (Linsley and Ledbetter,1993). A second CTLA-4 ligand has now been identified (B7.2) which may also be important in naive CD4 T cell activation. Crosslinking antigen receptors on naive CD4 T cells does not induce proliferation whereas this event will induce proliferation on antigen-experienced T cells (Luqman and Bottomly,1992). However naive T cells will proliferate when the CD80 molecule on APC is ligated by CD28 expressed on the T cell (Linsley *et al*,1991a).

Hsa is a small glycoprotein which can also stimulate naive CD4 T cells (Liu *et al*,1992a). Hsa is expressed on B cells, activated T cells, monocytes, granulocytes, Langerhan's cells and thymocytes. It has been found that both CD28/CTLA-4 and Hsa are required for optimal CD4 T cell activation (Liu *et al*,1992b).

CD45 is another molecule that plays a critical role in T cell activation though it is not a co-stimulatory molecule. Purified CD45 has tyrosine specific phosphatase activity (Tonks *et al*,1988). The cytoplasmic region of CD45 consists of two protein tyrosine phosphatase domains which regulates the tyrosine phosphorylation and activity of both p56lck and p59fyn in T cells. Studies on several CD45 deficient T cell lines have demonstrated that the signal transduction process initiated by TCR ligation is interrupted in the absence of CD45. This implies that the inability to signal through the TCR in CD45-deficient lymphocytes is due to improper regulation of tyrosine phosphorylation (Trowbridge and Thomas,1994). T cells change their CD45 isoform expression during and subsequent to activation. How the expression of different CD45 isoforms influences cellular function remains to be defined.



### 1.5.7 Cytokines

Signal transduction events that occur during antigen recognition by T cells produce a variety of cytokines. It is the co-ordinate production of cytokines which is crucial for the regulation of an immune response. In 1986, Mosmann and Coffman described a panel of murine CD4 T cell clones that revealed distinct patterns of cytokine production and effector function. This led to the subdivision of effector CD4 T cells into distinct subsets based on their cytokine profiles (reviewed in Janeway *et al*, 1988; Mosmann and Coffman, 1989; Kelso *et al*, 1991).

The IL-2,  $\gamma$ IFN and TNF $\beta$  producing clones were designated Th1 type and the IL-4, IL-5, IL-6 and IL-10 producing clones Th2. Cells of both types can produce IL-3, TNF $\alpha$  and GM-CSF. It has since become clear that the type of immune response generated following antigen challenge is dependent on the pattern of cytokines produced. The different patterns of the two types of Th cells leads to distinct effector functions. Th1 cells are adept at macrophage activation and immunoglobulin selection for IgG2a and IgG3 isotypes. These isotypes mediate antibody-dependent cytotoxicity and complement activation. Th2 cells favor activation of mast cells and eosinophils. They also stimulate B cell growth differentiation and isotype switching to cells producing IgE and IgG1. In general, Th1 cells generate cellular immunity against intracellular pathogens while Th2 cells promote the development of humoral responses that direct effector functions using specific antibodies against extracellular pathogens.

This mouse Th1/Th2 classification has since been validated for humans using antigen specific human CD4 T cell clones (Romagnani, 1991). CD4 T cell clones that exhibit Th1 or Th2 like profiles accumulate in tissues or peripheral blood of patients in disease states. Most CD4 Th clones specific for antigens of the nematode *Toxocara canis* exhibited a Th2 profile of cytokine secretion whereas the majority of Th cell clones generated from the same donors specific for purified protein derivative (PPD) from *Mycobacterium tuberculosis* showed a Th1 profile (reviewed in Romagnani *et al*, 1994). A distinction from mice is the production of IL-10 by both human Th types (Del Prete *et al*, 1993).

The polarization of Th1 and Th2 responses is influenced by the cytokine milieu in which they develop. The initial event that determines cytokine producing phenotype is the differentiation of naive precursor cells (Thp) under the influence

of factors present at the time of primary stimulation. Naive Thp cells produce IL-2 which acts as an autocrine growth factor. Thp cells progress into the intermediate Th0 stage in which cytokines of both Th1 and Th2 are produced. In the murine system these cells then terminally differentiate into Th1 or Th2 type cells under the influence of cytokines produced upon repeated antigen stimulation (Street *et al*,1990).

Once induced, the subsequent expansion of Th subsets is facilitated and regulated by subset specific cytokines (reviewed in Trinchieri,1993; Paul and Seder,1994). The dominant cytokines responsible for Th1 and Th2 differentiation are IL-12 and IL-4 respectively. IL-12 produced by macrophages in response to bacteria or parasites is an obligatory factor for Th1 generation and proliferation. IL-12 acts directly on Th1 cells and indirectly by inducing  $\gamma$ IFN production by T and NK cells.  $\gamma$ IFN has a positive feedback effect by positively inducing further IL-12 production from macrophages. The effect of  $\gamma$ IFN in enhancing IL-12 may be mediated by its ability to block endogenous IL-10 production by macrophages (Chomarat *et al*,1993). The presence of  $\gamma$ IFN and the subsequent down-regulation of IL-4 promotes the development of CD4 Th1 cells (Hsieh *et al*,1993). On the other hand IL-4 is required for the development of a Th2 response by inhibiting IL-2 receptor expression and  $\gamma$ IFN production. The Th2 cell products, IL-10 and IL-4 have a negative feedback effect by inhibiting IL-12 production (D'Andrea *et al*,1993). In order to determine whether IL-12 and IL-4 directly initiate Th1 and Th2 development in naive T cells, Hsieh *et al* (1993) showed that naive CD4<sup>+</sup> T cells derived from mice transgenic for an anti-ovalbumin TCR, were induced by ovalbumin, to develop into Th1 type cells in the presence of IL-12 and Th2 type cells in the presence of IL-4.

Macrophages, NK cells and mast cells have all been implicated in the directed development of the Th phenotype (Kullberg *et al*,1992). NK cells are a major source of  $\gamma$ IFN *in vivo* (Gately *et al*,1994). In addition to CD4 T cells, mast cells have been shown to synthesize significant quantities of IL-4 (Bradding *et al*,1993). NK cells and macrophages produce  $\gamma$ IFN for Th1 development, while mast cells provide IL-4 for Th2 type responses.  $\gamma\delta$  T cells may also contribute to the cytokine milieu that influences the differentiation of antigen specific CD4 T cells.  $\gamma\delta$  T cells have been shown to produce cytokines appropriate to the Th response much earlier than CD4 cells following infection (Ferrick *et al*,1995). Macrophages, mast cells, NK cells and  $\gamma\delta$  T cells appear to play a significant role in directing the immune

response.

The influence of IL-4 and IL-12 in the development of functional T helper subsets *in vivo* has been established using the mouse model of *Leishmania major* infection (Heinzel *et al*,1989). Strains of mice that are highly susceptible (Balb-c), develop poor cell mediated immunity to the parasite and produce Th2 type cytokines. In contrast, resistant mouse strains such as C3H develop Th1 responses. Susceptible Balb-c mice can become resistant when treated with anti-IL-4 antibodies (Sadick *et al*,1990) or administering IL-12 (Heinzel *et al*,1993) near the time of parasite inoculation. Recently it has been reported that treatment with IL-12 in combination with a leishmanicidal drug induces healing in *L. major* infected mice. This healing was associated with a switch from Th2 to Th1 response (Nabors *et al*,1995).

The immune response is directed by cytokines, however locally active hormones (Daynes *et al*,1991) antigen dose and antigen specific recognition can also control the functional outcome of immunity. Peptide density on APCs has been predicted from MHC haplotype and the cell mediated immunity was found associated with the haplotype displaying low ligand density (Murray *et al*,1994). Ligand density may be important in controlling the functional outcome of the Th response.

The Th1/Th2 analysis has also been extended to CD8 T cell clones which similarly subdivide on their cytokine secretion patterns (Salgame *et al*,1991). This was demonstrated for CD8 T cells specific for *Mycobacterium leprae* derived from skin biopsies of patients with leprosy. Th1 CD8 clones were derived from healed lesions of patients with tuberculoid leprosy. Th2 CD8 T cell clones were derived from patients with lepromatous leprosy. Similarly CD8 T cell clones generated from Kaposi's sarcoma skin lesions of patients with AIDS secrete IL-4 and IL-5 but not  $\gamma$ IFN and show diminished cytolytic activity (Del Prete *et al*,1994). Mouse and rat CD8 T cells in *in vitro* culture have also been shown to produce IL-4 when stimulated with IL-2, IL-4 and ConA/anti-CD3 (reviewed in Kemeny *et al*,1994; Seder and Le Gros,1995).

## **1.6 Antigen presentation by MHC class II on T cells**

Antigen presentation has been considered a specialized function of professional APCs (macrophages, B cells and dendritic cells) that constitutively



express class II. The functional significance of class II expression on activated human T cells is unclear. The ability of T cells to present soluble antigens is limited by the lack of an efficient mechanism of antigen capture but T cells have been shown to present peptide antigens *in vitro*.

Receptor-mediated interactions with antigens are required by T cells to capture and present soluble antigens effectively. This has been demonstrated for mouse immunoglobulin bound to the T cell surface, gp120 of HIV which binds CD4 (Lanzavecchia *et al*,1988) and for hepatitis B virus which binds to the transferrin receptor (Franco *et al*,1992). Peptides or whole antigen covalently coupled to anti-CD4 mAbs have also been shown to be taken up processed and presented by T cells (Wyss-Corray *et al*,1991). Thus T cells are not processing deficient but their antigen presenting capacity is limited by their inefficiency in capturing soluble antigens.

Though presentation of whole antigen by T cells is dependent on antigen capture, T cells are able to present denatured antigens or peptides. The highly purified native myelin basic protein (MBP) cannot be processed and presented by T cell clones, however T cells can present peptides of MBP to stimulate epitope specific autologous T cell clones (LaSalle *et al*,1991). This has also been demonstrated for influenza virus haemagglutinin specific T cell lines (Hewitt and Feldman,1989). The inability of T cells to process whole antigens would imply that T cells have no APC function *in vivo*. T cell peptide presentation may represent an *in vitro* phenomenon of T cell clones binding excess peptide directly to class II molecules (LaSalle *et al*,1991). There has been a report of uptake of soluble antigen by pinocytosis (Barnaba *et al*,1994), but these experiments did not include controls to exclude uptake independent presentation. Using physiologically activated class II positive sheep T cells in contrast to *in vitro* T cell lines no induction of antigen-specific T cell proliferation was found even when using digested antigen (Hopkins *et al*,1993). Overall activated class II positive human T cells have the ability to present antigens when bound to receptors on the T cell. The significance of their ability to present peptides is controversial.

That activated T cells present antigens has been demonstrated in humans (Lanzavecchia *et al*,1988) and sheep (Hopkins *et al*,1993) by their capacity to stimulate allogeneic T cells in mixed lymphocyte reactions (MLR). This can be expected as the alloantigen forms an integral part of the polymorphic structure of

the class II molecule. LaSalle *et al* (1991), showed that T cells can present the autoantigen, myelin basic protein (MBP) peptides, to autologous T cells in an MHC class II restricted fashion. The activation of autoreactive T cells may lead to the induction of autoimmune diseases. Much of the recent work on the functional significance of class II on activated T cells suggest an immunoregulatory function in T-T cell interactions. Broeren *et al* (1994), have reported direct evidence of T cell regulation of activated T cells presenting TCR fragments in the rat. It was demonstrated that T cells process and present their own TCR proteins to anti-TCR peptide specific MHC class II restricted CD4<sup>+</sup> T cells. T cell regulation of activated T cells also underlies the principal of T cell vaccination. In patients with multiple sclerosis (MS) there is an increased frequency of activated MBP reactive T cells (Allegretta *et al*,1990). Vaccination of MS patients with irradiated autologous MBP reactive T cells induces an MHC restricted T cell response to the MBP-reactive T cells (Zhang *et al*,1993). It was postulated that this response was regulated by a clonotypic network whereby peptides of the TCR variable region were presented by T cells themselves. Indeed the up-regulation of class II on T cells is coincident with internalization/down-regulation of the TCR (Valitutti *et al*,1995). Therefore the presentation of TCR fragments on activated T cells may maintain peripheral tolerance.

This phenomenon may also be linked to the anergizing signals reported for antigen presenting T cells. T cells primed with superantigen or by peptide presenting T cells become unresponsive to further antigenic stimuli (O'Hehir and Lamb,1990; LaSalle *et al*,1992) whereas activation by B cells does not alter the function of the reactive clones. Antigen presentation by T cells appears to down-regulate the immune response and results in clonal anergy. Thus T-T cell interactions may have important immunoregulatory function in the maintenance of peripheral tolerance through the induction of clonal anergy.

Activated human T cells express the costimulatory molecule B7 after repeated stimulation *in vitro*. B7 is also found on isolated T cells from HIV infected individuals (Wyss-Corray *et al*,1993). However not all activated T cells express detectable amounts of B7 (Pichler and Wyss-Corray,1994). The significance of B7 expression on T cells has yet to be ascertained.

The signal transduction capabilities of T cell class II has also been examined. Odum *et al* (1991) have shown that mAb to MHC class II molecules induce activation

of tyrosine kinases and mobilization of intracellular calcium in activated human class II positive T cells. Crosslinking class II with submitogenic amounts of anti-CD3 induces IL-2, IL-3,  $\gamma$ IFN and TNF $\alpha$  secretion by T cells (Spertini *et al*,1992). A more recent study, has shown that crosslinking class II molecules on human T cells enhances IL-2 sensitivity. It was further shown that DQ as opposed to DR mAb were unable to trigger this effect (Odum *et al*,1993).

The differential expression of class II isotypes on activation of T cells with a predominance of DR followed by DP and DQ has also been documented (Oshima and Eckels,1990a). Class II isotypes are differentially expressed on human T cells and appear to have different sensitivities to IL-2 based on their proliferative response, suggesting different roles in the regulation of T cell responses. Similarly in sheep there is a predominance of DR expression over DQ. Using T cells drawn from the various immunological compartments, variations in their cell surface phenotype correlated with activation states such that DQ is thought to be a marker of recent activation events while DR is a marker of memory (Hopkins *et al*,1993).

In summary class II molecules on human T cells have been shown to participate in MLR, autoantigen presentation, anergy induction and signal transduction. The most exciting possibility is the presentation by a T cell of idiotypic TCR peptides in the context of its own MHC class II molecules which may form a basis for specific anti-idiotypic T-T cell interactions.

### **1.7 Immunological memory**

It has long been recognized that the immune system holds a memory response to previous encounters with antigen. Individuals who survive diseases such as measles, mumps, chickenpox and whooping cough rarely suffer twice. The first contact clearly imprints some memory so that the body is prepared to repel any later invasion by that organism, a state of immunity is established. Compared to primary encounter with antigen, the secondary response is more rapid and effective. The memory response is dependent on T and B cells, the cells which recognize the antigen. Both T and B cells maintain and are responsible for the generation of memory. In this section the memory response of T lymphocytes is discussed.

Much research has been conducted on delineating cell surface markers which

relate to the activation state of the T cell. Initial studies in humans found that T cells could be divided into distinct populations based on expression of two isoforms of CD45, CD45RO and CD45RA\B\C (in human,mouse and rat) and adhesion molecules (reviewed in Mackay,1993). T cells that expressed CD45RA expressed low levels of adhesion molecules while CD45RO T cells expressed increased levels of adhesion molecules. Furthermore, T cells bearing the low molecular weight (CD45RO) isoform represented primed T cells and transferred specific responses based on their ability to proliferate to recall antigen (Sanders *et al*,1988). Thus it was established that CD45RA marked naive antigen inexperienced T cells while CD45RO delineated the memory T cells. Analysis on the distribution of these phenotypes throughout life found that the memory phenotype was rare in young life but became more conspicuous with age (DePaoli *et al*,1988; Ernst *et al*,1990). This led to the belief that the change in CD45 isoform expression represented an antigen driven unidirectional maturation event.

This concept has since been challenged by findings that CD45 isoform expression can readily interconvert. It was first discovered that T cell populations of the low molecular weight isoform transferred into athymic nude rats reverted to the high molecular weight isoform (Bell and Sparshott,1990). Several other studies have substantiated this finding. Mitogen stimulated CD45RO human T cell lines re-express the CD45RA isoform *in vitro* with time (Warren and Skipsey,1991). More recent data from Sparshott and Bell (1994) demonstrates that there is rapid interconversion of CD45R isoforms (<6 hours). Their evidence suggests that CD45RC expression in the rat reflects a dynamic state in which T cells switch back and forth between a resting (CD45RC<sup>+</sup>) and a more active state (CD45RC<sup>-</sup>). This has led to the current understanding, that there are no markers of memory but only markers of activation. On encounter with antigen T cells become activated, acquire markers, proliferate and become hyperreactive to antigen. After a brief phase, the stimulated cells revert to a resting phase and lose the markers but remain present in expanded numbers (Mitchison,1992; Sparshott and Bell,1994).

Though CD45 can readily interconvert, it still has value in defining different cell phenotypes. Evidence from CD45 deficient T cell lines demonstrate that CD45 is an absolute requirement for TCR signalling (reviewed in Trowbridge and Thomas,1994) and there is further evidence demonstrating that CD45 isoforms regulate signal transduction through the TCR differentially (Chui *et al*,1994). The

use of CD45 expression in conjunction with adhesion molecule expression provides a better definition of T cell phenotypes. T cells which have not encountered antigen since their exit from the thymus, express high levels of CD45RA/B/C (in human, mouse and rat respectively) L-selectin and low levels of CD44. Effector T cells which have been stimulated by antigen express a reciprocal phenotype with low levels of CD45RA/B/C (or high CD45RO) low levels of L-selectin and high levels of CD44. In addition effector/memory type cells have higher levels of several adhesion molecules such as ICAM-1, LFA-1, CD2, LFA-3 and VLA-4 (Sanders *et al*,1988; Mackay *et al*,1990).

An interesting approach recently reported (McHeyzer-Williams and Davis,1995) uses the T cell receptor sequences of responder cells as a memory marker. It was shown that certain sequences predominated in the secondary response while others disappeared, suggesting a selective preservation or expansion of specific T cell clones. The change in repertoire seen may represent a selection for T cells expressing a receptor with greater specificity for the antigen-major histocompatibility complex i.e. the memory response.

#### **1.7.1 Lifespan of naive and memory T cells**

The lifespan of both naive and memory T cells is controversial (reviewed in Gray,1993; Matzinger,1994; Sprent and Tough,1994). Because the cell surface markers used to define phenotypes are variably regulated, it is difficult to assess the precise functional phenotypes of T cells. For example when bromodeoxyuridine (BrdU), a DNA precursor is administered to thymectomized mice a proportion of the naive phenotype (CD45RB, L-selectin, Pgp-1) T cells were found to divide (Tough and Sprent,1994) implying that the surface phenotype of T cells does not correlate exactly with functional phenotype. However, because there is no definitive marker for memory/naive T cells, the cell surface phenotypic division is still used to define the temporal activation status of T cells.

Adoptive transfer experiments have demonstrated that naive phenotype T cells can survive for long periods in the host (Sprent *et al*,1991). Tough and Sprent's (1994) studies on thymectomized mice found with BrdU labelling that the naive phenotype T cells remain in interphase for prolonged periods. Thus it has been suggested that naive T cells are potentially long-lived. The longevity of these virgin cells allows the immune system to continue to mount primary responses to



new antigens even in advanced age.

If memory resides in long-lived cells, the presence or absence of antigen should be irrelevant. However in experiments transferring memory phenotype cells without antigen, these cells lost their function after a couple of months. In these adoptive transfer experiments memory type T cells were dependent on the presence of antigen for survival (Gray and Matzinger, 1991). Investigations on the lifespan of both CD45RO and CD45RA T cells in patients after radiotherapy show rapid loss of unstable chromosomes from the CD45RO but not the CD45RA pool (Michie *et al*, 1992). Immunological memory appears to reside in a population with a more rapid rate of division. Memory phenotype T cells in the sheep (CD45R) have also been noted as short-lived (Mackay *et al*, 1990). Tough and Sprent's studies using BrdU labelling in thymectomized mice, found that a proportion of the memory phenotype T cells divided rapidly while others remained in interphase for weeks. Long-term memory T cells have been shown by recent findings that murine cytotoxic T cells, which confer protection to viral antigen, survive for years in the apparent absence of persisting antigen (Lau *et al*, 1994; Hou *et al*, 1994). These results favor a model whereby both short term memory and long term memory T cells exist. Short term memory cells require constant restimulation to ensure survival of memory while long term memory cells survey the tissues until they make contact with specific antigen. Short term memory T cells may be maintained by the slow release of antigen from dendritic cells, keeping these cells in a constant state of activation. It is also possible that CD8 T cells exhibit long term memory while CD4 T cells require constant antigen stimulation for longevity.

### **1.7.2 Migration of memory and naive T cells**

Because the frequency of antigen specific lymphocytes is low, continuous migration through the tissues of the body enhances the likelihood of cells of appropriate specificity coming into contact with individual antigen (reviewed in Picker, 1992; Gray, 1993; Mackay, 1993).

Much of this work has been carried out using the ovine cannulated lymphatic model. This model allows access to *in vivo* fractionated cell populations from distinct lymphoid compartments. The vast majority of lymphocytes migrate through the body by passing from the blood to secondary lymphoid tissues (lymph

nodes, Peyer patches, gut associated lymphoid tissue) by way of specialized high endothelial venules (HEVs) (Hall,1974). Lymphocytes return to the blood via the efferent lymph ducts and the thoracic duct. Small numbers of lymphocytes also leave the blood in peripheral tissues. Cells that enter tissues eventually accumulate in the afferent lymphatic ducts. Within this system different migration routes have been proposed for naive and memory phenotype T lymphocytes (Mackay,1991).

T cells draining the skin into the afferent lymphatics are mostly memory type. This is consistent with observations that T cells with the memory phenotype are highly enriched in inflammatory sites (Pitzalis *et al*,1988). Inflammatory stimuli result in large numbers of memory type lymphocytes crossing inflamed endothelia (Pitzalis *et al*,1991; Picker,1993). T cells within efferent lymph derived from the blood via high endothelial venules (HEVs) predominantly express the naive phenotype (Mackay *et al*,1990). More recent studies show a tissue specific migration route of activated T cells to the skin and gut (Mackay *et al*,1992 a & b). Thus it appears that naive cells enter lymph nodes through high endothelial venules, while cells with the memory phenotype leave the blood at other sites and enter the lymph nodes through lymphatic drainage from these tissues. In the lymph nodes antigens arrive via the afferent lymph and presentation to naive T cells by dendritic cells and macrophages occurs (Bujdoso *et al*,1989). Thus naive T cells are able to screen a large drainage area in a short time. In contrast the migration of memory T cells in non-lymphoid tissues allows them to carry out their effector function directly and reach the lymph nodes via the afferent lymph. The tropism exhibited by memory T cells allows them to survey tissues that possibly frequently encounter the specific antigen.

Primary antigen challenge to lymph nodes produces only minor changes in lymph flow and cell output. In contrast secondary challenge results in cell shutdown and increased lymphocyte output (Hall and Morris,1965; Mackay *et al*,1990; Hopkins *et al*,1993). Presumably antigen delivered on primary challenge is presented by dendritic cells and macrophages to the naive T cells in the node thus establishing a memory response to the specific antigen. The increased traffic in secondary challenge is correlated by increased traffic of the memory phenotype T cells and blastogenesis of B cells. This increased traffic of memory type T cells serves to place functionally potent T cells at sites of antigen deposition.

A number of lymphocyte and endothelial cell adhesion molecules have been



identified which define where cells adhere and migrate. The adhesion molecules which facilitate differential migration include the selectins (L-selectin, E-selectin and P-selectin) and their ligands, the immunoglobulin superfamily cell adhesion molecules (ICAM-1, ICAM-2, VCAM-1) and the integrins (LFA-1, the  $\alpha 4$ ,  $\alpha 5$ ,  $\alpha 6$  and  $\beta 1$ ,  $\beta 2$ ,  $\beta 7$  heterodimers). The  $\alpha 4 \beta 1$  integrin has been found to be one of the principal adhesion molecules for lymphocyte extravasation to inflammatory sites (van Kooyk and Figdor, 1993). In sheep, skin memory phenotype T cells express high levels of the  $\alpha 6$  and  $\beta 1$  integrins in contrast low levels are expressed by gut memory phenotype T cells (Mackay *et al*, 1992a&b). L-selectin on the other hand has been implicated in lymphocyte binding to lymph node HEV (Gallatin *et al*, 1983). Naive T cells are L-selectin positive enabling them to enter lymph node through the blood while memory T cells lack this receptor and reach the lymph node through afferent lymph (Mackay, 1991).

A multistep hypothesis (Butcher, 1991) proposes that lymphocyte extravasation is the result of (a) primary adhesion (activation independent), (b) lymphocyte activation and (c) activation dependent adhesion. Various chemoattractants and cytokines have been implicated as activation factors in this process.

## 1.8 Aim of thesis

The aim of the work presented in this thesis was to investigate the functional significance of class II expression on sheep T cells. A proportion of resting T cells express class II and on activation there is increased expression of the products of both DR and DQ genes on T cells. This is evident in each of the T cell subsets (CD4, CD8 and  $\gamma \delta$ ). The function of class II positive T cells is not fully understood.

Firstly, it was necessary to define subgroup specific MHC class II molecules. The NH<sub>2</sub>-terminal amino acid sequence of the molecules recognized by two monoclonal antibodies (mAbs) was carried out to confirm their locus specificity. This allowed their comparison with homologues identified in humans and mice. Using defined mAbs the differential expression of class II on lymphocyte subsets was analysed in the various immunological compartments of the cannulated sheep. Expression following *in vivo* activation was also examined.

Class II antigens (DR, DQ) on sheep T cells may be described as activation

markers, however their relationship with other markers of activation is unknown. Work was conducted to correlate class II antigen expression on sheep T cells with other known activation markers. A separation technique was employed to fractionate the T cell populations into CD4<sup>+</sup>, CD8<sup>+</sup> and  $\gamma\delta$ <sup>+</sup> (T19) T cell pools. Activation marker expression and class II antigen expression was then correlated directly on each of these subsets by two colour flow cytometry.

The different products of the MHC class II loci on T cells have been shown to vary in their signal transduction capabilities (Odum *et al*, 1993). Signal transduction events that occur during antigen recognition by T cells produce a variety of cytokines. The cytokines secreted subdivide T cells into Th1 or Th2 types. This subdivision can delineate their functional role. For further insight into the function of the class II isotypes on T cells, cytokine profiles of DR<sup>+</sup> T cells was compared with that of DR<sup>-</sup> T cells.

Thus by analysing class II expression with relation to other markers of activation combined with the analysis of cytokine patterns of the different class II antigens, it was hoped to achieve a greater understanding of the functional importance of MHC class II positive T cells.

## **CHAPTER 2 Materials and Methods**

### **2.1 Materials**

#### **2.1.1 Chemical reagents and plasticware**

Materials used in the preparation of this thesis were supplied by Sigma Chemical Company, Poole, Dorset, U.K. and BDH Laboratory Supplies, Poole, England unless otherwise stated. Nunc, Denmark supplied the sterile tissue culture ware. All radioisotopes were supplied by Amersham International plc., Buckinghamshire, England.

#### **2.1.2 Animals**

Blackface sheep were obtained from the Moredun Research Institute, Edinburgh. They were kept in cages and given food and water *ad libitum*. Animal surgery was performed by Dr John Hopkins and all animal inoculations and blood sampling were performed by suitably licensed colleagues.

#### **2.1.3 Media for cell culture**

Cell cultures were maintained in RPMI 1640 (Gibco Biocult, Uxbridge, U.K.) supplemented with 2mM L-glutamine, 100 U/ml benzylpenicillin and 100 U/ml streptomycin, 500 mM 2-mercaptoethanol, 2.5mM Hepes and 10% foetal calf serum (Flow Laboratories, Hertfordshire, U.K.). Wash media was RPMI containing 1% foetal calf serum.

#### **2.1.4 Monoclonal antibodies**

The monoclonal antibodies (mAbs) used were from stocks held within the department. A summary of the mAbs used and their specificities for the sheep is given in Table 2.1

### **2.2 Methods**

#### **2.2.1 Immunizations and secondary *in vivo* challenge of animals**

Sheep were antigenically primed by intradermal injection of 1 mg ovalbumin in complete Freund's adjuvant and by subsequent injections of 1mg ovalbumin in sterile PBS. Secondary *in vivo* challenge of primed animals 4-6 weeks after primary immunizations was by intradermal injection of 100µg ovalbumin given in 1 ml sterile PBS containing 1% patent blue dye into several

sites around the drainage area of cannulated lymphatic vessels.

**Table 2.1** Monoclonal antibodies recognizing ovine (ov) leukocyte molecules.

<u>mAb</u>	<u>Specificity</u>	<u>Isotype</u>	<u>Reference</u>
ST197	ovT19 (WC1)	IgG2b	Maddox, 1986
CC15	ovT19 (WC1)	IgG2a	Clevers <i>et al</i> , 1990
ST4	ovCD4	IgG1	Maddox <i>et al</i> , 1985
SBU-T4	ovCD4	IgG2a	Maddox <i>et al</i> , 1985
E95	ovCD8	IgM	Maddox <i>et al</i> , 1985
SBU-T8	ovCD8	IgG2a	Maddox <i>et al</i> , 1985
VPM 8	ovIgG1	IgG1	Jones, 1988
VPM30	ovB cell	IgM	Naessens & Howard, 1991
VPM 19	ovMHC class I	IgG1	Hopkins <i>et al</i> , 1990
SW73.2	pan MHC class II $\beta$	Rat IgG2b	Hopkins <i>et al</i> , 1986
VPM54	ovMHC DR $\alpha$ chain	IgG1	Dutia <i>et al</i> , 1994
VPM36	ovMHCDQ $\alpha$ chain	IgG1	Dutia <i>et al</i> , 1994
HMT3.1	anti-human CD3	IgG1	Kubo <i>et al</i> , unpublished
F10/150/39	ovLFA1	IgG1	Mackay <i>et al</i> , 1990a
L180/1	ovLFA3	IgG1	Hunig, 1985
IL-A111	anti-bovine CD25	IgG1	Naessens <i>et al</i> , 1992
73B	ovCD45RA	IgG1	Mackay <i>et al</i> , 1990a
35A-1	ovCD2	IgG1	Giegerich <i>et al</i> , 1989
25-32	ovCD44	IgG1	Mackay <i>et al</i> , 1988
25-91	ovCD5	IgG1	Mackay <i>et al</i> , 1985
218	ov $\alpha$ 4 integrin	IgG2b	Sanchez-Madrid <i>et al</i> , 1983
Du1-29	ovL-selectin	IgG1	Mackay <i>et al</i> , 1992
VPM64	ovFc $\gamma$ III	IgG1	Gupta, 1994
VPM65	ovCD14	IgG1	Gupta, 1994
VPM21	ovBDV	IgG1	Dutia <i>et al</i> , 1990b
VPM22	ovBDV	IgG2b	Dutia <i>et al</i> , 1990b
MS121	ovIgGA	IgG2a	unpublished
2 $\gamma$ SH	ovIgG2	rat IgG2b	unpublished

### 2.2.2 Collection of lymph

Surgical techniques for the cannulation of efferent lymphatic vessels were as described by Hall (1967). Pseudoafferent lymphatic cannulations were as those described for the prefemoral lymphatic except that the node had been excised at least 6 weeks previously. During this time the afferent vessels anastomose with the efferent duct remaining after removal of the node (Hopkins *et al*, 1986). Cannulations were allowed at least 3 days to stabilize following surgical procedures. Lymph collections were made from fully conscious animals into sterile siliconized bottles containing 200 units heparin.

### 2.2.3 Preparation of cells

#### 2.2.3.1 Peripheral blood leukocytes (PBL)

Peripheral blood leukocytes were isolated from heparinized blood by  $\text{NH}_4\text{Cl}$  lysis as described by Mishell and Shiigi (1980). 10 mls of blood was added to 35 mls lysis buffer (0.16M  $\text{NH}_4\text{Cl}$ , 0.017M Tris, pH7) warmed to 37°C.

Clarification of the solution indicates lysis of the red blood cells. The cells were centrifuged at 200g for 5 minutes and washed twice in PBS. Peripheral blood leukocytes were also prepared by defibrinating blood end over end with glass beads. The defibrinated blood was then layered over Lymphoprep (Nygaard, Oslo, Norway) and centrifuged at 850g for 20 minutes. When dealing with large quantities of defibrinated blood, red blood cells were lysed by hypotonic shock before layering over lymphoprep. 10mls of blood was added to 15mls sterile water and immediately reequilibrated with the addition of 1.5 mls of 10X sterile PBS.

#### 2.2.3.2 Lymphocytes

Lymphocytes from efferent and afferent cannulations were washed twice in PBA (1%BSA, 0.1% Na-azide, PBS) by centrifugation at 200g for 5 minutes for use in immunofluorescent analysis and washed twice in wash media for use in *in vitro* proliferation assays.

#### 2.2.3.3 Alveolar macrophages

Lungs recovered from the abattoir were rinsed with ice-cold Hanks Buffered Salt Solution (HBSS), (1mM  $\text{CaCl}_2$ , 5mM KCl, 0.5mM  $\text{KH}_2\text{PO}_4$ , 0.5mM  $\text{MgCl}_2$ , 0.5mM  $\text{MgSO}_4$ , 0.14M NaCl, 1mM  $\text{Na}_2\text{PO}_4$ , 5mM Glucose, 0.02M Hepes, 0.01% phenol red, pH7). Using a funnel down the trachea, HBSS was poured into both intact lungs and the lungs gently massaged to release macrophages. Cells were

harvested by centrifugation at 800g for 15 minutes at 4°C, washed twice with HBSS and resuspended in 10mls RPMI. Erythrocytes were lysed by hypotonic shock, 15mls water was added to the cell suspension, followed immediately by 1.5mls 10x PBS to restore isotonicity. The cells were washed, resuspended in culture media at  $2 \times 10^6$ /ml in T75 flasks and allowed to adhere to the plastic flasks for 2 hours in the incubator. Non-adherent cells were poured off and replaced with fresh culture media and stimulated with bacterial lipopolysaccharide (from *Salmonella abortus equi*) at 10µg/ml. Cells were directly harvested into RNAzol B at various time points following stimulation.

#### 2.2.3.4 Cell fixation

Cells were suspended at  $1 \times 10^8$  per ml in PBA. An equal volume of 1% paraformaldehyde was added to the cell suspension, incubated for 5 minutes then washed.

### 2.2.4 Immunochemical Techniques

#### 2.2.4.1 Caprylic acid precipitation

Immunoglobulin was isolated from ascitic fluid by caprylic acid treatment (Russo *et al*, 1983). 25µl glacial acetic acid was added per ml of ascites to bring the pH to 5.0. 50µl of caprylic acid was added per ml of ascites and stirred at room temperature for 30 minutes, centrifuged at 13000g for 5 minutes then placed at 4°C for 30 minutes to remove the solidified caprylic acid. The purified Ig was then dialysed into several changes of 0.1M sodium bicarbonate buffer pH 8.4. Protein concentration was determined by spectrophotometer, whereby a reading of 1 absorbance unit at 280nm is the equivalent of 715 µgIgG.

#### 2.2.4.2 Biotinylation

Purified Ig was coupled to biotin (biotinamidocaproate N-hydroxysuccinimide ester) (1mg/ml in DMSO) at a biotin/IgG ratio of 75µg/mg. The suspension was rotated end over end for 4 hours at room temperature. The biotinylated Ig was dialysed against PBS containing 20% (v/v) glycerol and titrated for flow cytometric use.

#### 2.2.4.3 Immunodiffusion

This technique was based on that developed by Ouchterlony (1967). 1%

(w/v) agarose in PBS-0.05% (w/v) Na-azide gels were prepared. Sera was added to the central well and ovalbumin at 0.5mg/ml added to the outer wells. The plates were incubated in a humid chamber overnight. Plates were examined and stained with Coomassie Brilliant Blue G250.

#### 2.2.4.4 Cytospin and Leishmann Staining

Cells at a concentration of  $10^4/100\mu\text{l}$  were spun onto glass slides at 1200rpm. Slides were allowed to air dry and then stained with Leishmann stain for 1 minute followed by a wash in Leishmann buffer pH6.8. Slides were allowed to air dry then mounted in DPX.

### 2.2.5 Immunofluorescence Staining

#### 2.2.5.1 Immunofluorescent staining and flow cytometry

Antibodies supplied as saturated supernatants were used undiluted, whereas antibodies obtained from ascitic fluid were diluted in PBA to give optimal fluorescent intensity. The optimal working dilution of the various conjugates was determined by titrating against optimal dilutions of the primary antibody so that the mean and mode of fluorescence distribution coincided.

For single colour immunofluorescent staining,  $10^6$  cells were incubated with  $50\mu\text{l}$  of mAb at appropriate dilution for 30 minutes at room temperature. Unbound antibody was removed by washing in PBA. Cells were resuspended in  $50\mu\text{l}$  of FITC anti-mouse Ig (Scottish Antibody Production Unit, Carlisle, U.K.). After a 30 minute incubation the cells were analysed per sample with dead cells excluded on the basis of forward light scatter.

Dual colour immunofluorescence requires that each antibody is detected by a distinct fluorochrome. Immunoglobulin prepared from ascitic fluid of the anti-cell phenotype antibodies (CD4, CD8, VPM30 and CC15) was biotinylated. These biotinylated mAbs were detected using streptavidin-phycoerythrin (SA-PE) (Amersham,UK). The second antibodies were used in conjunction with the relevant Ig subclass-specific FITC conjugated anti-mouse Ig antiserum (The Binding Site, Birmingham, UK).

Cells were stained simultaneously with  $25\mu\text{l}$  of the appropriate dilution of both antibodies. After 30 minutes incubation at room temperature, cells were washed twice and incubated for a further 30 minutes with dilutions of SA-PE and FITC-antiimmunoglobulin before finally washing twice and analyzing. Dual staining using SW73.2 required that each staining step be done sequentially, first staining



with SW73.2, followed by an anti-rat-PE conjugate (Serotec, Oxford, U.K.), followed by second antibody and anti-mouse FITC conjugated antisera.

#### 2.2.5.2 Immunofluorescent analysis

All fluorescence analysis was carried out using a Becton Dickinson FACScan cell analyzer. Live gating using forward scatter (FSC) and side scatter (SSC) parameters were used to distinguish the different cell populations in afferent lymph, efferent lymph and blood. The gate used for small lymphocytes is shown in Figure 2.1. The fluorescence profile of a population of cells is presented as a histogram of fluorescent intensity versus relative cell number. Linear amplification was used for the physical parameters (FSC,SSC) and logarithmic amplification used for the fluorescence parameters (FL1,FL2). FL1-FL2 and FL2-FL1 compensation was used to optimize the two-colour analysis. Antibody positive cells were gated by reference to biotinylated normal mouse Ig at 10 $\mu$ g/ml or irrelevant anti-rat or mouse antibody followed by FITC conjugated anti-serum IgG. Calculations of percentage positive cells were obtained by subtracting any residual percentage staining on the gated negative control from all positive staining.

#### 2.2.6 Cell purification

Magnetic activated cell sorting (MACS) was used to either negatively or positively select cells. The cellular content of afferent lymph, efferent lymph and PBL from which cell populations were isolated is shown in Table 2.2.

##### 2.2.6.1 MACS selection for flow cytometric use

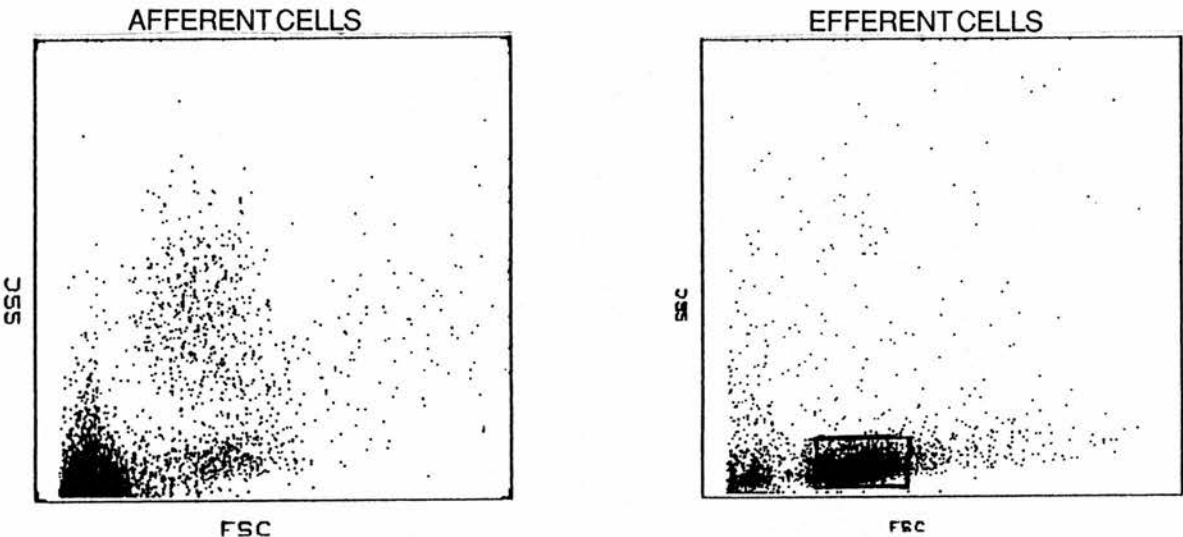
When separating cells for flow cytometric use, cells were first fixed in 0.5% paraformaldehyde (described in 2.2.3.4). Cells were counted and suspended in PBA and the appropriate titre of biotinylated mAb for 40 minutes. Cells were then washed and resuspended at 10<sup>7</sup> cells/90 $\mu$ l plus 10 $\mu$ l MACS streptavidin microbeads and incubated for 15 minutes before washing again.

Cells were applied in suspension to the ferromagnetic stainless steel wool column in a strong magnetic field. Columns were incubated with 1% BSA for 15-60 minutes prior to use to saturate non-specific binding sites. Cell suspensions were allowed to pass through the column under the force of gravity and the speed of elution altered by changing the size of elution needle. For negative selection a low flow speed is necessary (~1ml/min) whilst for positive selection the flow rates are less critical. Positively selected cells were repassaged through the column four times to achieve high purity. The effluent was collected as the negative fraction and

labelled cells were eluted outside the MACS magnetic field with the needle removed from the column, as the positive fraction.

**Figure 2.1**

Forward scatter (FSC) and side scatter (SSC) profile of afferent lymph cells and efferent lymph cells. The gated region on the efferent profile represents a small lymphocyte gate.



**Table 2.2**

The percentage of CD4<sup>+</sup>, CD8<sup>+</sup>, T19<sup>+</sup> and B cells in efferent lymph, afferent lymph and PBL is shown.

	EFFERENT	AFFERENT	PBL
CD4 <sup>+</sup>	48.7±3.1 (n=7)	54.7±3.8 (n=7)	36.8±15.0 (n=5)
CD8 <sup>+</sup>	16.2±2.8 (n=7)	11.1±2.4 (n=9)	11.0±6.5 (n=5)
T19 <sup>+</sup> (γδ)	8.0±3.1 (n=7)	9.1±1.3 (n=7)	7.1±2.7 (n=4)
B cell	14.8±7.3 (n=5)	16.3±7.8 (n=7)	26.7±6.0 (n=6)

The purity of the cell populations was assessed by (1) immunofluorescently labelling with monoclonal antibodies specific for various subset markers followed by isotype specific second layer reagents or (2) by incorporating streptavidin conjugated phycoerythrin (SA-PE) with the MACS streptavidin microbead incubation step at 5µl SA-PE per ml of cell suspension.

#### 2.2.6.2 MACS selection for in vitro use

The same method was used except that cells were not fixed but kept on ice during this procedure. Tissue culture wash media was used throughout.

### 2.2.7 Transmission Electron Microscopy

Ovine T lymphocytes were fractionated into a CD4, CD8,  $\gamma\delta$  and B cell positive fraction and a CD4, CD8,  $\gamma\delta$  and B cell negative fraction using the MACS (described in 2.2.6.1). Cells were prepared for electron microscopy by the staff at the Veterinary Preclinical Department, University of Edinburgh. Cells were fixed overnight in 0.1M sodium cacodylate pH7.3 and 3% glutaraldehyde buffer. Cells were washed three times in 0.1M sodium cacodylate pH7.3 and treated with 1% osmium tetroxide made up in 0.1M sodium cocodylate pH7.3. Samples were dehydrated through graded acetone solutions and embedded in araldite. Ultrathin sections were cut, stained with 50% uranyl acetate (in ethanol) and Reynolds lead citrate for examination in a Phillips TEM 400 operating at 100kV.

### 2.2.8 Proliferation assays

#### 2.2.8.1 Ovine peripheral blood mononuclear cells (PBMC)

PBMC prepared from both antigen primed and non-primed sheep were resuspended in culture medium and  $10^5$  cells cultured in 96-well round bottomed microtest trays (Gibco) with various concentrations of antigen (ovalbumin). Cultures were made to a final volume of 200µl and established in triplicate. Concanavalin A (Con A) at 5µg/ml was used as a positive control and human serum albumin (HSA) at the same concentrations as ovalbumin as a negative control. The proliferation assays were incubated for 5 days at 37°C in 5% CO<sub>2</sub>. <sup>3</sup>H-thymidine at 1µCi/well was added for the last 4 hours of culture. Cultures were harvested onto glass filter using a Harvester-96 (Tomtec, CT,USA) and the <sup>3</sup>H-thymidine incorporation was measured by beta spectroscopy.

## 2.2.8.2 Proliferation assays for cytokine analysis

### 2.2.8.2.1 Proliferation with Con A

Leukocytes collected from blood or efferent lymph were cultured in T25 flasks at  $2 \times 10^6$  cells/ml with  $5\mu\text{g/ml}$  Con A. Cells were harvested at various time points and cell pellets lysed directly for RNA extraction. In some experiments the lymphocytes were first fractionated using the MACS into MHC class II DR positive or negative T cell subpopulations .

### 2.2.8.2.2 Proliferation with ovalbumin

Using efferent cells for proliferation assays, autologous PBMC were irradiated to act as antigen presenting cells. PBMC prepared from ovine blood were suspended in RPMI and 1% FCS at a concentration of  $2 \times 10^6$  cells/ml and irradiated using a cobalt-caesium source. The amount of radiation that prevented subsequent proliferation of PBMC was 3000 rads. Total efferent cells were MACS selected into MHC class II DR positive and negative fractions and  $10^7$  cells set up in culture with an equal number of irradiated PBMC. Ovalbumin was added at  $500\mu\text{g/ml}$ . Cells were harvested at various time points after stimulation and lysed directly for RNA extraction.

## 2.2.9 Purification of MHC class II for amino acid sequencing

### 2.2.9.1 Column Preparation

Immunoglobulin was purified by caprylic acid precipitation as described previously (2.2.4.1) and dialysed into several changes of 0.1M phosphate buffer pH7.2. The protein concentration was determined by spectrophotometry and coupled to Affigel 10 (Bio-Rad, Hertfordshire, U.K.) for 4 hours at  $4^\circ\text{C}$ . Unbound sites were blocked with 1M ethanolamine pH8.0 for 1 hour. The antibody linked Affigel 10 was then washed with TNT (20mM Tris-HCl pH 8.0, 150mM NaCl, 0.5% Triton X100).

### 2.2.9.2 Lysate Preparation

Ovine class II was purified from sheep spleen (obtained from the local abattoir). Spleen tissue was teased from the capsule, homogenized in ice cold HBSS using an electric blender. Cells were pelleted by centrifugation at 800g for 15 minutes and lysed in TNT containing 0.2mM phenyl methyl sulphonyl flouride (PMSF). Nuclei were removed by centrifugation for 15 minutes at 800g. The

lysate was clarified by centrifugation for 90 minutes at 3000g followed by filtration through glass fibre filters (Millipore, Ireland). The lysate was pumped onto a normal mouse Ig Affigel 10 column followed by either a VPM36 or a VPM54 Affigel 10 column. The column was washed through with TNT buffer followed by 15mM triethanolamine-HCl, pH 8.0, 0.5% sodium deoxycholate and eluted with 15mM triethanolamine-HCl pH11.3, 0.5M NaCl, 0.5% sodium deoxycholate. Eluted fractions were neutralized with respect to pH by addition of 0.5M triethanolamine pH7.9. Protein containing fractions were identified by spectrophotometer at 280nm and peak fractions pooled. The columns were reequilibrated with PBS-0.05% sodium azide.

#### 2.2.9.3 Isolation of monomers for amino acid sequencing

Isolation of the individual  $\alpha$  and  $\beta$  chain monomers of MHC class II was accomplished with the Applied Biosystems Model 230A HPEC, an automated micropreparative electrophoresis system. Separation was achieved on 10% polyacrylamide gels [30% (w/v) acrylamide, 0.8% (w/v) piperazine diacrylamide(PDA)] in Tris-phosphate pH7.5. The separated eluted  $\alpha$  chains of VPM36 and VPM54 were centrifuged onto a polyvinylidene difluoride (PVDF) membrane using Prospin cartridges (Applied Biosystems, Warrington, U.K.). Sequencing was carried out at the SERC Protein Sequencing Facility, University of Aberdeen, U.K. on an Applied Biosystems pulsed liquid analyser.

### 2.2.10 Protein Chemistry

#### 2.2.10.1 Preparation of cell samples for SDS polyacrylamide gel electrophoresis (SDS-PAGE)

Cells were counted and pelleted by centrifugation at 800g for 5 minutes and lysed in TNT containing 0.2mM PMSF at a concentration of  $5 \times 10^7$  cells/ml, for 30 minutes on ice. Nuclei were removed by centrifugation at 13000g for 10 minutes. The supernatant was then acetone precipitated by the addition of 5 volumes of ice-cold acetone-1mM HCl and overnight incubation at  $-20^{\circ}\text{C}$ . The precipitate was recovered by centrifuging for 10 minutes at 13000g.

#### 2.2.10.2 SDS-PAGE

Proteins were separated according to size by electrophoresis using the method of Laemmli (1970). Protein fractionation was on polyacrylamide gels with a Tris-glycine buffer containing SDS using the Bio-Rad mini-protean II system

apparatus. The separating gel consisted of 12% polyacrylamide [30% (w/v) acrylamide, 0.8% (w/v) bisacrylamide], 375mM Tris-HCl pH 8.7, 0.1% (w/v) SDS with 0.05% (w/v) ammonium persulphate and 0.05% (v/v) TEMED added to polymerize the gel. This gel was then overlaid with a stacking gel consisting of 3% polyacrylamide 125mM Tris-HCl pH 6.8, 1% SDS, 0.05% ammonium persulphate and 0.1% TEMED. Samples were mixed with an equal volume of sample buffer [0.125M Tris-HCl, pH 6.8, 20% (v/v) glycerol, 2% (w/v) SDS, 0.04% (w/v) bromophenol blue], boiled for 3 minutes then added to stacking gel. A set of proteins of known molecular weights (Pharmacia, Herts, U.K.) were used as size markers. Gels were run at 200V until the dye front reached the bottom of the gel.

#### 2.2.10.3 Silver staining of gels

Gels were fixed in 50% (v/v) methanol, 10% (v/v) acetic acid for 15 minutes, 5% methanol, 7% acetic acid for 30 minutes and then 10% (v/v) glutaraldehyde for 30 minutes. Gels were then washed thoroughly in several changes of deionised water over several hours. After washing, gels were stained with 0.1% (w/v) silver nitrate solution for 15 minutes, rinsed briefly with water then developed with a 3%(w/v) sodium carbonate 0.02% (v/v) formaldehyde solution. Colour development was stopped by the addition of solid citric acid. Gels were rinsed well, fixed in 10% Ilfofix (Ilford, Cheshire) for 1 minute, washed then dried at 80°C under vacuum.

#### 2.2.10.4 Immunoblotting

After electrophoretic separation on SDS-PAGE, proteins were transferred to nitrocellulose membrane (Hybond C, Amersham) using a semi-dry electroblotter (Ancos, Denmark). The gel was placed on top of the membrane and sandwiched between blotting paper soaked in blotting buffer (48mM Tris, 39mM glycine, 20% methanol) current at 120mA was applied for 45 minutes. Following transfer, marker tracks were stained with 0.5% amido black and the remaining membrane blocked in 5% (w/v) non-fat dried milk powder (Cadburys, Stafford, U.K.) in PBS before overnight incubation in primary antibody. Blots were washed in several changes of PBS-1% non-fat dried milk powder over 60 minutes at room temperature. Bound primary antibody was detected by incubating for 60 minutes with an anti-mouse whole IgG molecule biotin conjugate (Boehringer Mannheim, East Sussex, U.K.) followed by alkaline phosphatase conjugated to streptavidin (Boehringer Mannheim). Excess reagent was removed by several changes of buffer over 60 minutes at room temperature. The immunoblots were developed with



0.17mg/ml nitro-blue tetrazolium and 80µg/ml 5-bromo-4-chloro-3-indolylphosphate in 0.1M Tris-HCl pH9.5 and 3.5mM MgCl<sub>2</sub>. Colour development was stopped by washing in water.

### **2.2.11 Molecular Biology Techniques**

In the manipulation of nucleic acids care was taken in keeping preparations free of nucleases. All handling was done wearing gloves, sterilized ware was used and solutions kept on ice. Dimethylsulphoxide (DEPC) treated water was used for RNA work (0.2% (w/v) DEPC added to water, incubated for 2 hours, then autoclaved).

#### **2.2.11.1 Purification of RNA**

Total RNA was prepared by the method of Chomczynski and Sacchi (1987) modified by Cinna/Biotech (RNAzol B method, Biogenesis Ltd., Bournemouth, U.K.). The cell pellets were lysed directly by the addition of RNAzol B (1ml/10<sup>7</sup> cells). RNA was solubilized by passing the lysate 5-6 times through the pipette. Chloroform was added to 10% and samples shaken vigorously for 15 seconds before placing on ice for 5 minutes. The suspensions were centrifuged at 13000g for 15 minutes and the upper aqueous phase transferred to a fresh tube with the addition of an equal volume of ice cold isopropanol. Samples were stored on ice for a minimum of 15 minutes, when using low numbers of cells the isopropanol step was carried out overnight. The RNA was precipitated with another 15 minute centrifugation. The RNA was then washed using 75% ethanol. RNA pellets were finally resuspended in 50µl DEPC treated water and stored at -70°C. The concentration of RNA was estimated by measuring the absorbance at 260nm of aliquots diluted 1:100 in water and applying the equation :-  $RNA = A_{260} \times 100 \times 40\text{ng/ml}$ . Absorbance readings at 280nm were also measured to check the sample purity. Pure preparations of RNA have A<sub>260</sub>/A<sub>280</sub> ratios of 2.0. If the ratio is less than this value, the sample is contaminated with protein or phenol and the calculated concentration of nucleic acid is not then reliable. The integrity of the RNA was checked by agarose gel electrophoresis when sufficient quantities of RNA was recovered.

#### **2.2.11.2 Synthesis of cDNA**

Single stranded DNA was synthesised by the method of Gubler and Hoffman (1983) from total RNA using a commercial kit (1<sup>st</sup> strand cDNA synthesis system, Amersham). Reactions were carried out according to manufacturers instructions using 1µg amounts of RNA per reaction. Oligo dT was used to prime from the 3'



poly(A)tail of mRNA. Incorporation of  $1\mu\text{Ci } \alpha^{32}\text{P-dCTP}$  in this reaction allowed the yield of cDNA to be estimated. The reactions were allowed to proceed at  $42^{\circ}\text{C}$  for 1 hour.

#### 2.2.11.2.1 Estimation of the amount of radioactivity incorporated into nucleic acid

On completion 10% of the reaction mix was diluted 10 fold and an equal volume spotted onto filter discs (Millipore, Herts, U.K.) in duplicate. One set of discs were washed in an ice-cold solution of 5% TCA followed by ice cold ethanol and finally in ice cold acetone. The discs were allowed to dry thoroughly. Scintillant was added and the percentage incorporation of radioactivity was measured using an LKB liquid scintillation counter. The unwashed filter measures the total radioactivity in the sample while the washed filter measures only the incorporated nucleic acid. The percentage of input radioactivity incorporated into DNA can be calculated, thus allowing the calculation of mass of cDNA synthesized. The yield of cDNA from each reaction was calculated as follows

$$\text{cDNA synthesized} = \frac{A \times B}{C} \text{ ng}$$

C

where A= the residue molecular weight of 1mole dNTP (350)

B= the total amount of dNTP incorporated

$$\frac{4 \times \% \text{incorporation}}{\text{nmoles of unlabelled dCTP incorporated}}$$

nmoles of unlabelled dCTP incorporated

C= the weight of input RNA

#### 2.2.11.2.2 Purification of cDNA for polymerase chain reactions (PCR)

The cDNA synthesised was diluted to  $100\mu\text{l}$  with TE buffer (10mM Tris-HCl, pH8.0, 1mM EDTA pH8.0) and phenol(pH8.0):chloroform (1:1) extracted. The aqueous phase was further extracted with chloroform. The cDNA was precipitated with 16% 4M Ammonium acetate and 64% ethanol at  $-20^{\circ}\text{C}$  for a minimum of 2 hours. The DNA was precipitated at  $13000g$  for 10 minutes and resuspended at  $1\text{ng}/\mu\text{l}$  from the estimation described above.

#### 2.2.11.3 Probes and Primers

Oswel DNA Service, Edinburgh, supplied all primers and probes. The sequence for all the primers and probes used in this thesis are listed in Table 2.3. Primers were designed not to have detrimental intra-annealing or inter-annealing characteristics.

**Table 2.3** Sequences of primers and probes.

Cytokine	Primer/probe sequence (S=sense, A=anti-sense ,P=probe)	Code Name	Size of PCR product
ATPasealpha	S5'-GCTGACTTGGTCATCTGC3' A5'-CAGGTAGGTTTGAGGGGAT AC 3' P5'-CATCCCCTGCTGGAAGACG GA ATT 3'	2 $\alpha$ + 2 $\alpha$ - $\alpha$ (3i) -	165
$\gamma$ -interferon	S5'-GAGGTTAGATTTTGGTGAC AG 3' A5'-GGGCCTCTCTTCTCAGAAA TT 3' P5'-GGCAGCTCTGAGAACTGG CAGGAC3'	G3107 G3108 G4151	303
IL-4	S5'-CACTGTCAGTGCAAATAGAG A5'-CCCTCATAATAGTCTTTAGC P.5'-TGGTCTGCTTACTGGTATG	G5287 G5289 G5290	416
IL-6	S5'-CGGGATCCATTGAAGGTAGGAT GAACTCCCGATTCAAGCG3' A5'-CGGGATCCTACTTCATCCGAAT AGCTCTC3' P5'-TTCCTCCAGAACGAGTTTGAG3'	053R 159R 805T	627
IL-10	S5'-ATGCCACAGGCTGAGAACC3' A5'-GTTCACAGAGAAGCTCAGT3' P5'-CAAGAGCAAGGCGGTGGAGC AGGT3'	E419 860X G1338	452

#### 2.2.11.4 Polymerase chain reactions

PCR reactions were set up in laminar flow hoods using aerosol barrier pipette tips (BioRad). Reaction mix volumes were 100µl overlaid with mineral oil. Reactions were performed using both buffers and enzyme supplied by Biogene Ltd, Bedfordshire, England. Reaction conditions were as follows :- 75mM Tris-HCl pH9, 20mM ammonium sulphate, 0.01% (w/v) Tween, 2.5mM MgCl<sub>2</sub>, 100µM dNTP's, 0.05-0.12µM of each primer and a standardized amount of substrate cDNA. An equal volume of water was added as a negative control. Prior to the addition of the enzyme the reaction was heated at 80°C for 5 minutes, then 0.33 of a unit of enzyme was added diluted in water. Automatic thermal cycling was performed on either a Techne PHC-1 machine or a Hybaid Omnigene machine using block control. PCR reactions involved 40 cycles of denaturation at 95°C for 36 seconds, 36 seconds at 53°C annealing temperature followed by an extension for 1.5 minutes at 75°C with a final extension at 75°C for 3 minutes. 10% of the DNA synthesized was analysed on agarose gel electrophoresis.

#### 2.2.11.5 Slot-blotting of PCR products

In a separate laboratory to that used for the preparation of samples for PCR, the products of PCR reactions were denatured and blotted onto nylon membrane (Hybond-N+, Amersham). 10µl of amplified DNA harvested at various cycle points was added to 100µl 0.4M NaOH -10mM EDTA in a 96 well microtitre plate and incubated for 10 minutes. The plate was then placed in a boiling waterbath for 3 minutes. Samples were transferred to nylon membrane previously soaked in water for 10 minutes via a Bio-Rad (BIODOT) manifold. 500µl of SDW was passed through the filter under vacuum followed by sample and finally washed through with 500µl of 0.4M NaOH-10mM EDTA. The DNA was immobilized onto the membrane by baking at 80°C for 1 hour.

##### 2.2.11.5.1 Prehybridization and hybridization of slot-blot

Membranes were prehybridized in 5mls hybridization solution [2xSSC (0.15M NaCl, 0.15M NaCitrate, pH7), 10x Denhardt's, 1% SDS, 200µg/ml salmon sperm DNA and 120µg/ml yeast RNA] in a Hybaid hybridization oven at 45°C. The appropriate probe was added to 3mls of fresh hybridization solution and membranes

were hybridized in the oven for a minimum of 3 hours at 45°C. Membranes were washed twice at room temperature in 2xSSC for 5 minutes with a final higher stringency wash in 0.5X SSC at 50°C for 1 hour. The blots were saran wrapped and exposed to XAR-5 scientific imaging film (Kodak) at room temperature and processed.

#### 2.2.11.6 Agarose gel electrophoresis of DNA

DNA samples were assessed by electrophoresis through 1-2% (w/v) agarose gels. Agarose was melted in TAE buffer (40mM Tris acetate pH7.8, 1mM EDTA) containing 0.5µg/ml ethidium bromide. Prior to loading samples, samples were mixed with 0.25 volumes of loading buffer [0.25% (w/v) bromophenol blue, 0.25% (w/v) xylene cyanol, 15% (w/v) ficoll, 5xTAE]. Gels were cast as horizontal slab gels and run in 1xTAE at 70V using an LKB 2013 miniphor electrophoresis unit. Gels were generally run for 1 hour and samples analysed using a UV transilluminator and photographed using a Mitsubishi video copy processor. Estimates of molecular size and mass of DNA bands were made by comparing their mobility with those of known standards (NBL, Northumberland, UK) run in parallel.

#### 2.2.11.7 Agarose gel electrophoresis of RNA

RNA gels were carried out under denaturing conditions. Samples were fractionated by electrophoresis on 25cm long horizontal slab gels of 1.5% agarose containing 18% formaldehyde and 20mM Na phosphate pH7. Samples were heated to 70°C for 15 minutes with 3 volumes denaturing buffer (60% deionised formamide, 24% formaldehyde, 20mM Na phosphate pH 7) then a 1:5 dilution of dye mix (30% ficol, 0.05% bromophenol blue, 100mM phosphate pH7, 100µg/ml ethidium bromide) was added before samples were loaded into individual wells and fractionated by electrophoresis initially for 15 minutes at 60V in Na phosphate. The buffer was then circulated and run at 40V overnight. Gels were further stained in 1µg/ml ethidium bromide for 1 hour prior to UV transillumination.

#### 2.2.11.8 Southern Blot

DNA fragments contained within agarose gels were transferred to nylon membrane (HybondN+, Amersham) using techniques based on the method of Southern (1975). Before capillary transfer to the membrane the DNA in the gels was depurinated in 0.4M NaOH for 20 minutes. Gels were then laid on a wick of

Whatman 3MM filter paper, connected to a reservoir of 0.4M NaOH and overlaid with a Hybond N+ membrane prewetted in water. The transfer of DNA was effected by a continual upward migration of buffer for 16 hours achieved by overlaying the membrane with 2 sheets of 3MM paper, topped with a wad of paper towels and compressed with a weight. DNA was immobilized by baking at 80°C for 1 hour.

#### 2.2.11.9 Radiolabelling of DNA

DNA oligonucleotides were end-labelled using the radioactive isotope  $^{32}\text{P}$ . The following was added to an eppendorf tube : 1 $\mu\text{l}$  of oligonucleotide, (OD 5-20/ml), 2.5 $\mu\text{l}$  10x kinase buffer(0.8M Tris pH7.5, 0.1M  $\text{MgCl}_2$ , 0.1M DTT), 18 $\mu\text{l}$  sterile water, 30 $\mu\text{Ci}$   $^{32}\text{P}$ - $\gamma$ -dATP and 1U polynucleotide kinase (Epicentre Technologies). The tubes were incubated at 37°C for 1 hour, the enzyme was then inactivated at 65°C for 15 minutes and the total volume brought to 200 $\mu\text{l}$  with sterile water.

## CHAPTER 3 The characterization of *Ovar* DR $\alpha$ and DQ $\alpha$ MHC class II molecules and their expression on T cells.

### 3.1 Introduction

The class II region in humans (HLA-DR) is composed of 3 loci HLA-DR, HLA-DQ, HLA-DP that are directly involved in antigen presentation. The HLA-DM loci encodes molecules which play a critical role in the antigen processing and transport to the endoplasmic reticulum (Morris *et al*,1994). The additional loci HLA-DN, -DO are also thought to be involved in antigen processing within the cell (Ting and Baldwin,1993). In the mouse the functional class II genes are grouped into two subregions (IA,IE) which are homologous to DQ and DR respectively (Kappes and Strominger,1988). Mice also have homologues to HLA-DM and DN (Karlsson and Peterson,1992). The functional subregions of class II contain A and B genes which encode  $\alpha$  and  $\beta$  proteins. The class II molecules expressed on the cell surface exist as a heterodimer of the  $\alpha$  (32,000) and  $\beta$  (28,000) glycoprotein chains non-covalently linked.

In recent years many studies have been carried out on the genetic arrangement of the ovine class II region (*Ovar*-D). Gene cloning and Southern blot hybridization studies indicate that *Ovar*-D region contains subregions homologous to the HLA-DR, DQ and DN regions of humans. No DP or DM regions have been identified (Scott *et al*,1987). Alleles within the DRA, DQA1, DQA2 and DQB regions have been identified (Fabb *et al*,1993; van Oorschot *et al*,1994), these regions in sheep are thus polymorphic. A feature of the sheep class II region which differs from the HLA class II region is the presence of a DY region (Wright *et al*,1994). The DY locus is represented by an A/B gene pair. Use of monoclonal antibodies has identified expressed DQ $\alpha$ , DQ $\beta$ , DR $\alpha$  and two DB $\beta$  class II molecules in the sheep (Dutia *et al*,1994).

The constitutive expression of MHC class II gene products is tightly regulated and is normally confined to professional antigen presenting cells. However, many cell types can be induced to express MHC class II molecules (reviewed in Glimcher and Kara,1992). For example, resting human T cells do not express class II but synthesize and express high levels upon activation. Oshima and Eckels (1990a), have shown differential expression of the class II locus products during activation of human T cells with a hierarchial dominance for DR, DP and DQ isotypes. In sheep, a percentage of resting T cells express class II and upon

activation increased expression occurs. Differential expression of the class II isotypes has also been reported in sheep (Dutia *et al*,1993a). The significance of the increased class II expression on activation of T cells is unclear.

On professional APC's class II expression serves to present antigens to T cells. On T cells the role of class II in presentation of antigen is not as clearcut. The antigen presenting capacity of class II on T cells has been found to be limited by their inefficiency in capturing antigens (Lanzavecchia *et al*,1988; Franco *et al*,1992; Wys-Coray *et al*,1991). Only antigens that directly bind to cell surface determinants are presented. Though presentation of whole antigen by T cells is dependent on antigen capture, human T cells have been shown to present denatured antigens or peptides. This has been demonstrated *in vitro*, using T cell clones (LaSalle *et al*,1991; Hewitt and Feldman,1989). It is possible that this may represent an *in vitro* phenomenon as when physiologically activated class II positive sheep T cells were used, Hopkins *et al* (1993), found no induction of antigen proliferative response to digested antigen. The ability of T cell class II to present antigens is somewhat limited, however antigens are presented. Presentation of antigens has been demonstrated in humans (Lanzavecchia *et al*,1988) and in sheep (Hopkins *et al*,1993) by their capacity to stimulate allogeneic T cells in MLR.

In addition to their function as antigen presenting structures, class II molecules on APCs can function as signal transduction elements. On murine and human B cells, crosslinking class II molecules induces protein tyrosine phosphorylation, increased cytosolic free calcium and activation of protein kinase C (reviewed in Julius *et al*,1993). Class II expression on T cells may also have a role other than antigen presentation. On activated human T cells, mAb to class II molecules induces activation of tyrosine kinases and mobilization of intracellular calcium (Odum *et al*,1991). Induced secretion of IL-2, IL-3,  $\gamma$ IFN and TNF $\alpha$  by T cells with class II and CD3 molecules crosslinked has also been reported (Spertini *et al*,1992). A more recent study has shown that the different class II isotypes show different sensitivities to IL-2 induction (Odum *et al*,1993). DR as opposed to DQ molecules have enhanced sensitivity to IL-2. Class II isotypes are selectively expressed on human T cells and their differing sensitivities to IL-2 induction suggest differing immunoregulatory roles.

The data presented in this chapter forms an introduction to class II isotype



expression on sheep T cells. Data in successive chapters will focus on the functional significance of class II positive ovine T cells. To study the expression of MHC class II on ovine T cells it was necessary to define sub-group specific MHC class II mAbs. Amino-terminal sequencing of the antigens recognized by two mAbs was carried out to confirm their locus specificity and allow their comparison to homologues in humans. Using the defined mAbs, the differential expression of class II subtypes in lymphocyte subsets was analysed from various immunological compartments. Ovine T cells circulating in afferent and efferent lymph have been identified phenotypically as memory and naive type cells respectively, while T cells of the peripheral blood contain a mixture of both phenotypes (Mackay *et al*,1990). By analysing the expression of class II in each of these compartments an overall picture of class II expression on phenotypically distinct T cells can be assessed. Use of the cannulated sheep lymphatic model allows access to *in vivo* fractionated cell populations from the distinct lymphoid compartments.

The differential expression of class II products following *in vivo* activation of efferent T cells was also examined. The efferent duct of a single peripheral lymph node can be cannulated and lymph collected over extended periods of time. Antigen challenge to the lymph node drainage area allows the subsequent analysis of draining *in vivo* activated T cells. Because primary antigen challenge is not associated with kinetic and phenotypic alterations in class II expression by ovine T cells (Hopkins *et al*,1993), only responses after secondary antigenic challenge were studied.

The aims of the work described in this chapter were

- (a) to determine the NH<sub>2</sub>-terminal amino acid sequence of the class II  $\alpha$  chain recognized by the mAbs, VPM54 and VPM36 in order to establish their MHC class II locus specificities,
- (b) to correlate the expression of class II subtypes with expression of T cell subset specific surface markers, in afferent lymph, efferent lymph and peripheral blood from sheep, and
- (c) to analyse the expression of class II subtypes on CD4<sup>+</sup>, CD8<sup>+</sup> and T19<sup>+</sup> T cells after *in vivo* activation of efferent lymph.

## 3.2 Results

### 3.2.1 Selection of DR and DQ mAbs.

VPM36 and VPM54 were selected for these studies as DQ and DR  $\alpha$  chain specific mAbs respectively. VPM36 has been described by Dutia *et al* (1990). Further data on the reactivity of both mAbs was published recently (Dutia *et al*, 1993a & 1994). In order to establish conclusively the MHC class II locus specificities of these mAbs, NH<sub>2</sub>-terminal amino acid sequence analysis of the purified  $\alpha$  chains was carried out.

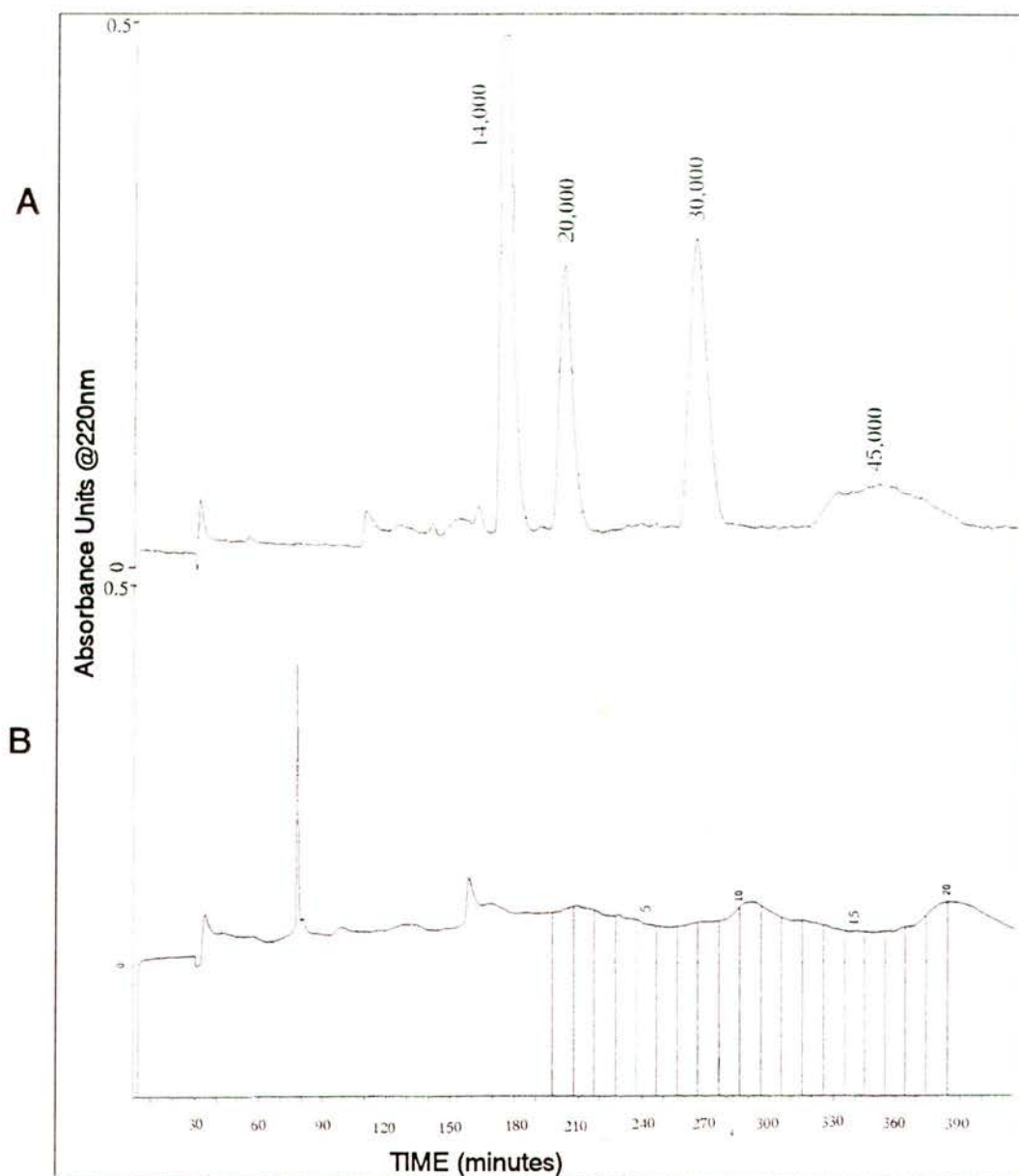
### 3.2.2 Purification of VPM36 and VPM54 $\alpha$ chains.

Sheep MHC class II molecules were purified from sheep spleens. The MHC class II heterodimers reacting with VPM36 and VPM54 were purified by immunoaffinity chromatography following the detergent solubilization of splenocytes. Separation of the  $\alpha$  and  $\beta$  chains was accomplished using an automated micropreparative electrophoresis system (HPEC, ABI, Warrington, U.K.). This instrument enables the collection of samples fractionated by SDS-PAGE. Sheep  $\alpha$  and  $\beta$  chains differ in molecular weight by approximately 2kDa. Conditions of electrophoresis were optimized to achieve such resolution as to separate these chains. This entailed varying the size of gels and the percentage of polyacrylamide used for the preparation of gels. 10% (10cm x 2.5cm) polyacrylamide gels proved optimal. Figure 3.1(A) is an elution trace of low molecular weight markers run on the HPEC under the conditions described. From this elution trace a range was predicted for fraction collection following the electrophoresis of class II molecules. An elution profile of a class II antigen is given in Figure 3.1(B). A silver stained SDS-PAGE gel of recovered  $\beta$  (28kDa) and  $\alpha$  (32kDa) chains from the heterodimer is given in Figure 3.1 (C). The individual fractions collected depict a stepwise increase of bands showing the changes due to glycosylation. From this data the  $\alpha$  chain fractions (13-15) were selected, transferred to PVDF membranes using a Prospin cartridge and sent for sequencing.



**Figure 3.1**

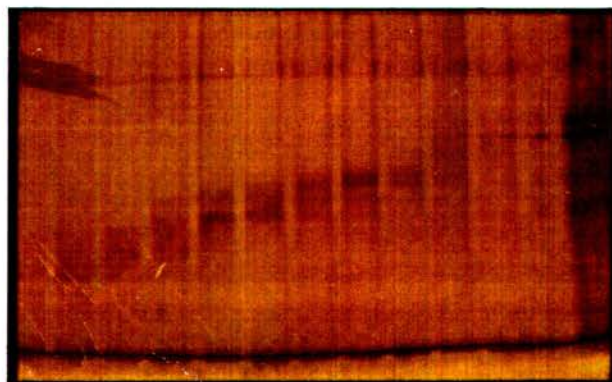
HPEC preparative separation of low molecular weight markers (A) and partially purified VPM54 antigen (B). The markings on the trace represent fractions collected. When analysed by slab-gel (C) fractions 7 - 15 show recovery of the pure  $\beta$  and  $\alpha$  chains from the heterodimer respectively.



Fraction No.

6 7 8 9 10 11 12 13 14 15 16 VPM54ag

**C**



### 3.2.3 Amino acid sequence of VPM36 and VPM54 $\alpha$ chains.

The sequence of the  $\alpha$  chain recognised by VPM36 compared with human and mouse  $\alpha$  chain sequences is given in Figure 3.2a. The amino acid sequence recognised by VPM36 is close to that of the amino acid sequence derived from cDNA sequence of human DQ $\alpha$  and mouse I-A $\alpha$  (Travers and McDevitt, 1987).

Comparisons with the amino acid sequences predicted from published cDNA sequences for *Ovar*-DQA1 and DQA2 (Fabb *et al*, 1993) indicate that the antigen recognized by VPM36 is more homologous to the predicted product of DQA1. However there is no conclusive data on the reactivity of VPM36 with the individual DQA gene products. VPM36 mAb may recognize both DQA gene products.

The sequence data of the  $\alpha$  chain recognised by VPM54 is given in Figure 3.2b along with an ovine DR $\alpha$  sequence (VPM38) determined by NH<sub>2</sub>-terminal amino acid sequencing (Dutia *et al*, 1993a) and an amino acid sequence predicted from cDNA sequence (Fabb *et al*, 1993). Each are homologous to human DR $\alpha$  and mouse I-E $\alpha$ . VPM54 $\alpha$  sequence is identical to the *Ovar*-DR $\alpha$  derived sequence published by Fabb *et al*, (1993) in the first 20 amino acids.

In conjunction with the ELISA data and 2-dimensional immunoblot data (Dutia *et al*, 1993a & 1994) it can be concluded that VPM36 and VPM54 represent DQ $\alpha$  and DR $\alpha$  sheep MHC class II locus products.

### 3.2.4 Expression of MHC class II *in vivo*.

Using a pan class II mAb (SW73.2) it has been shown that class II is expressed on resting T cells (Hopkins *et al*, 1986). To examine the expression of the individual locus products on functionally distinct lymphocytes, the staining of VPM36 and VPM54 on lymphocytes from different immunological compartments was analysed.

DQ and DR expression on CD4<sup>+</sup>, CD8<sup>+</sup> and T19<sup>+</sup> and B cells in afferent lymph, efferent lymph and peripheral blood was examined by two colour flow cytometric staining. Live gating on small lymphocytes excluded any contributions from blasting cells. Staining with these antibodies on T cells does not identify a discrete population rather a continuum indicating varying intensities of expression. The staining pattern is given in Figure 3.3. Both DR and DQ locus products were expressed on T cells at varying levels in each of the immunological compartments examined. B cell expression of class II was relatively homogenous in each compartment with all B cells expressing both DR and DQ.

Figure 3.2

(a) NH<sub>2</sub>-terminal sequence analysis of class II  $\alpha$  chain recognized by VPM36 compared with human DQ, mouse I-A  $\alpha$  and the predicted *Ovar* DQ $\alpha$ 1 and DQ $\alpha$ 2 chain sequences.

VPM36 $\alpha$	E . D I V A D H I	G	I L	
	A T Y G V N I Y Q T Y G P			
DQ $\alpha$	<u>E . D I V A D H V</u>	C	F	
	A S <u>Y</u> G V N L Y Q S Y G P			
I-A $\alpha$	<u>E D D I E A D H V</u>	S	I T	
	G V Y G T S V Y Q S P G D	F	V	
Ovar-DQ $\alpha$ 1	<u>E . D I V A D H I</u>	H	I	
	G T Y G V N V Y Q T Y G P			
Ovar-DQ $\alpha$ 2	<u>E . D I V A D H F</u>			
	G S Y G T E I Y Q S H G P	V I A D F		

(b) NH<sub>2</sub>-terminal sequence of the class II  $\alpha$  chain recognized by the mAb VPM54 compared with the NH<sub>2</sub>-terminal sequence of a sheep DR $\alpha$  (VPM38), sheep DR $\alpha$  sequence predicted from DNA, human DP $\alpha$ , human DR $\alpha$  and mouse I-E $\alpha$ .

VPM54 $\alpha$	I K E D H V I I Q A E F Y L N P E E S A A - M
Ovar-DR $\alpha$ (VPM38)	- <u>K E D</u> - T T <u>I Q A E F Y L N P E E S</u>
Ovar-DR $\alpha$	<u>I K E D H V I I Q A E F Y L N P E E S A E F M</u>
DR $\alpha$	<u>I K E E H V I I Q A E F Y L N P D Q S G E F M</u>
DP $\alpha$	<u>I K A D H V S T Y A A F V Q T H R P T</u>
I-E $\alpha$	<u>I K E E H V I I Q A E F Y L L P D K R</u>

Sequences underlined indicate identity with the sequences determined using VPM36 and VPM54, . indicates a gap inserted to maximise alignment, - indicates that residues could not be determined. Alternative residues indicate different alleles except for VPM36 $\alpha$  and VPM38 $\alpha$  where alternative residues indicate that more than one residue was present.

The percentage of T cells stained with MHC class II mAbs from a number of sheep are tabulated in Table 3.1. Data analysed from 5 sheep show DQ expression on 8% CD4<sup>+</sup> T cells, 12% CD8<sup>+</sup> T cells and 4% T19<sup>+</sup> T cells in efferent lymph. DR was expressed on 18% CD4<sup>+</sup>, 20% CD8<sup>+</sup> and 12% T19<sup>+</sup> efferent T cells (Table 3.1). Data on VPM54 and VPM36 staining on T cell subsets and B cells of afferent lymph and peripheral blood was obtained from two sheep. In the afferent lymph, levels of both DQ and DR expression were higher than in efferent lymph for each of the T cell subsets. DR and DQ expression on PBMC showed a similar pattern to that of efferent lymph on each of the T cell subsets. Two DQA genes both of which appear to be expressed are found in some but not all sheep (Scott *et al*, 1991). The reactivity of VPM36 with both gene products is not known. Thus two sheep is too small a sample size for interpretation of DQ expression. Only one DRA gene has been identified, however for statistical significance the sample size was again too small.

### **3.2 5 *In vivo* antigenic challenge.**

On activated human T cells DR cell surface densities are modulated over time while the density of DP and DQ molecules remain more constant. To assess the situation in ovine T cells, VPM54 and VPM36 staining on T cell subsets in efferent lymph at various time points following a secondary immune response was examined. Cells of the efferent lymph are described as phenotypically naive (Mackay *et al*, 1990) and so it was of interest to examine class II expression after activation. Changes in expression levels of class II do not occur during primary immune responses (Hopkins *et al*, 1993) and only secondary responses were examined.

The secondary response to ovalbumin was examined in 3 sheep. Representative data on VPM36 and VPM54 staining of T cell subsets is shown in Figure 3.4. Using the cannulated model of the sheep for functional studies regular lymph collection was difficult. As a consequence it was not possible to obtain data at equal time intervals for each animal examined.

Lymph cells were stained with anti-phenotypic cell surface mAb and either VPM54/VPM36 mAb. Live gating using FSC/SSC parameters was used to delineate small lymphocytes. Increased levels of DR and DQ expression were observed in each of the T cell subsets over 9 days. The levels returned to resting levels by day 12.

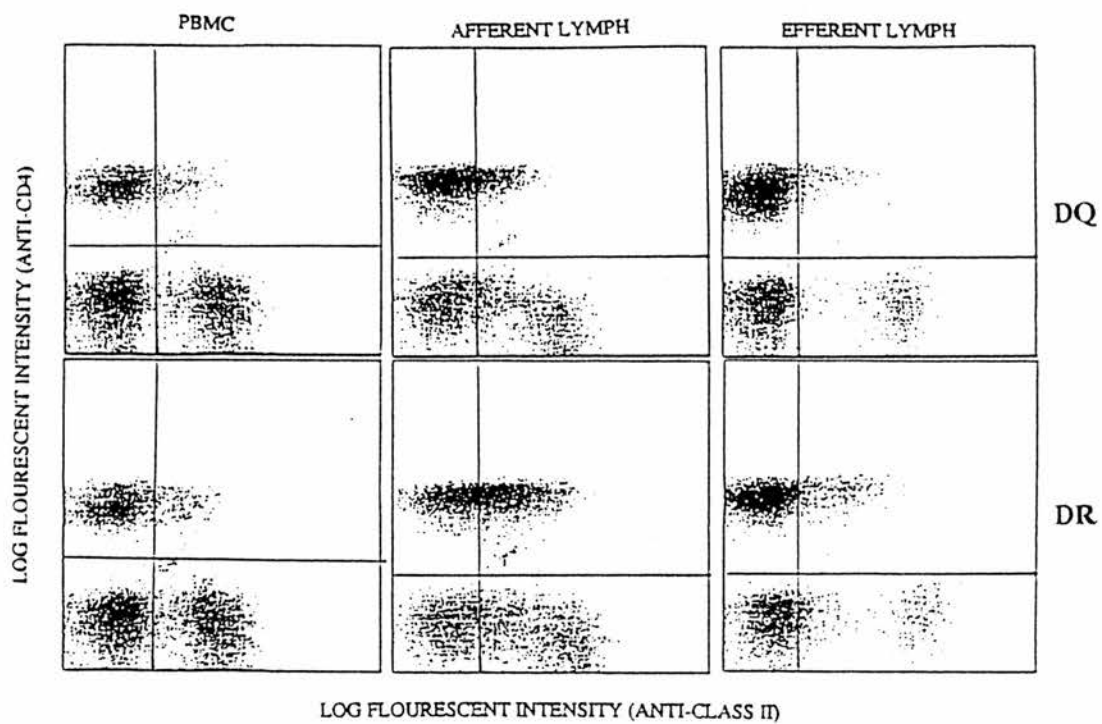


### Figure 3.3

Two colour flow cytometry analysis of lymphocytes from adult sheep peripheral blood (PBMC), afferent lymph and efferent lymph stained with anti-class II subgroup specific mAbs VPM36 and VPM54 and the anti-phenotypic mAbs, anti-CD4 (a), anti-CD8 (b), anti-T19 (c) and anti-B cell (d). The anti-phenotypic mAbs were coupled to biotin and detected with streptavidin-labelled phycoerythrin and the anti-class II mAbs detected with isotype specific FITC labelled anti-mouse Ig.

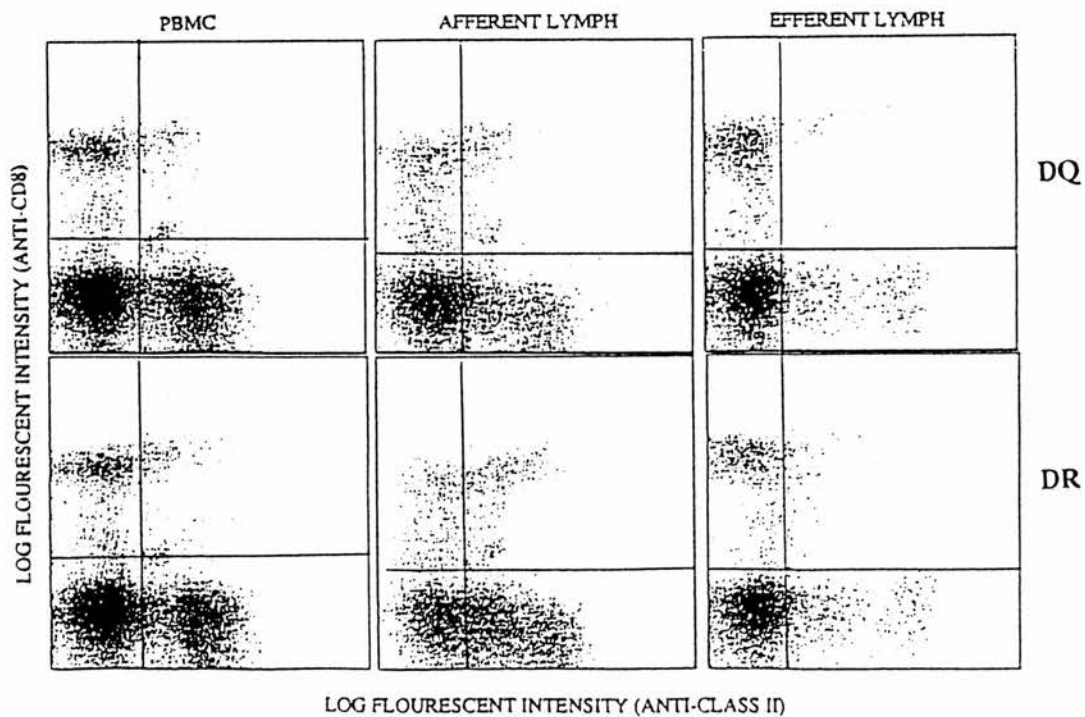
(a)

CD4 + T CELLS



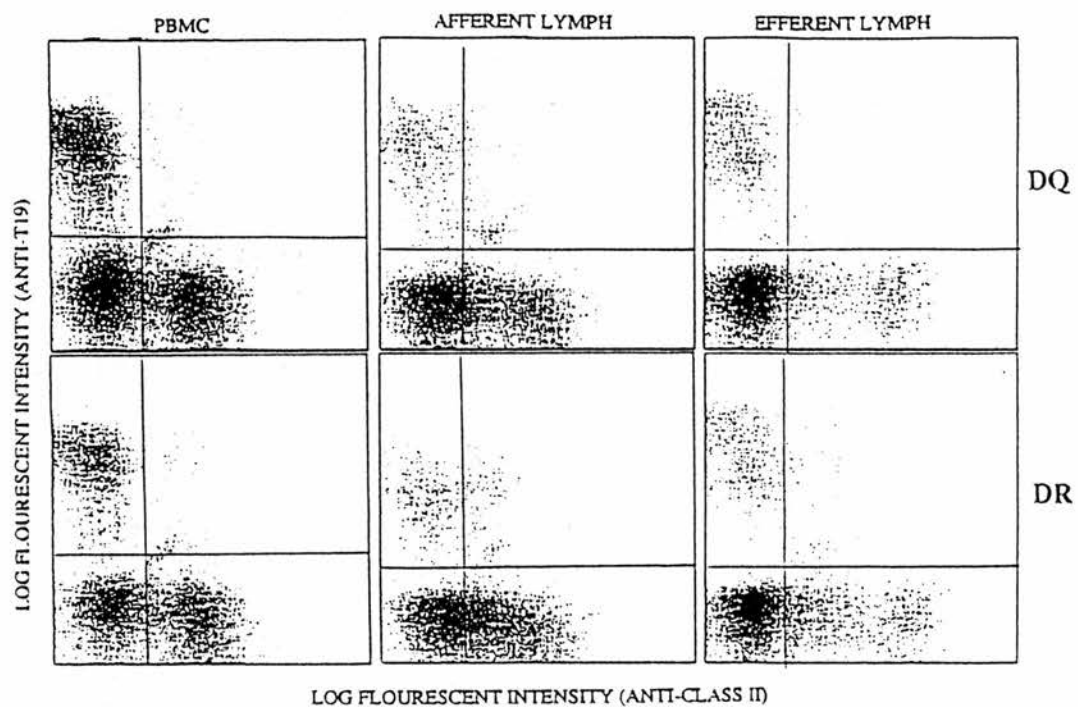
(b)

CD8 + T CELLS



(c)

T19 + T CELLS



(d)

B CELLS

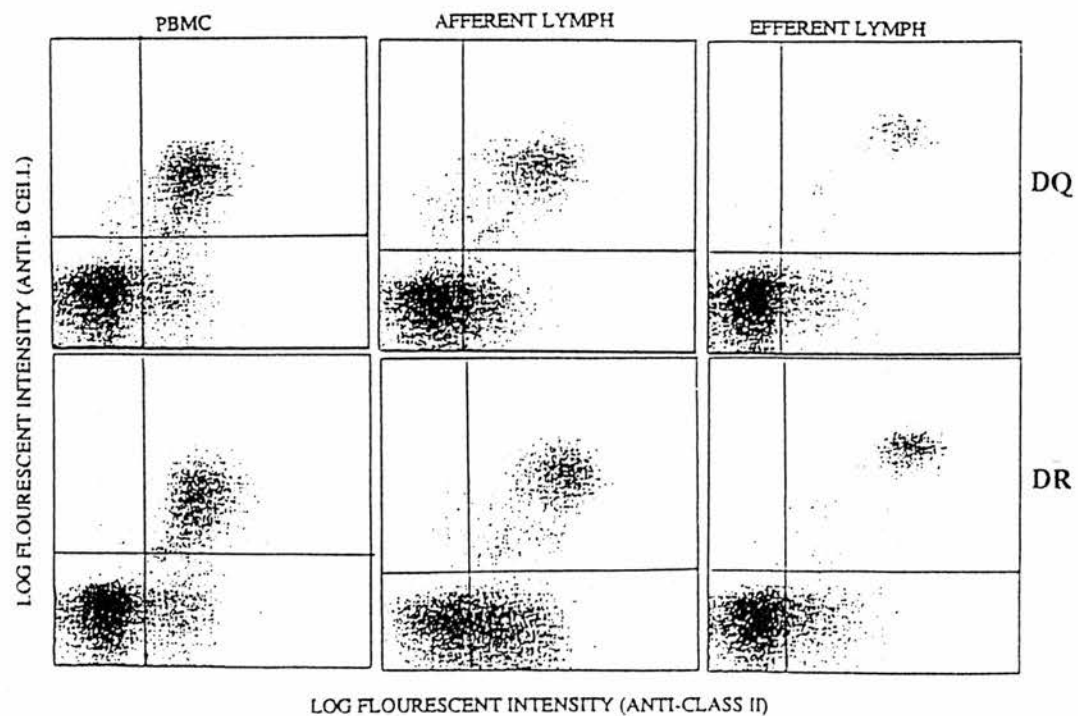


Table 3.1

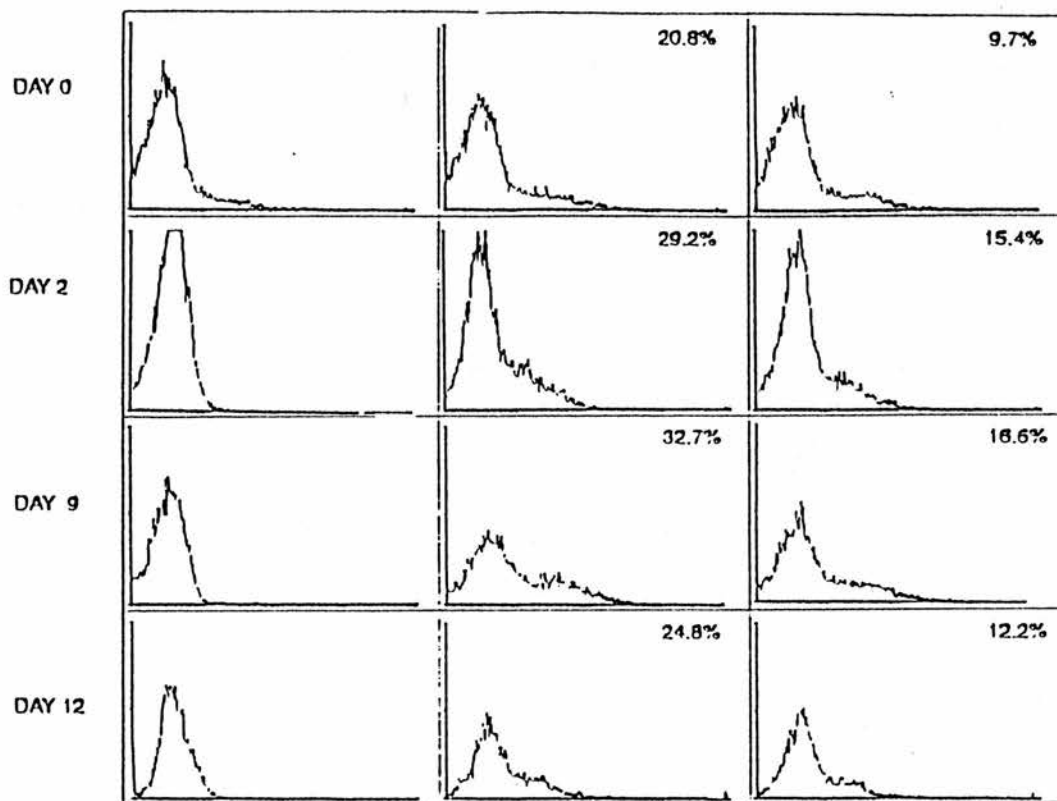
Percentage of T cells from different lymphoid compartments expressing MHC class II DQ and DR molecules.

		PBMC	AFFERENT LYMPH	EFFERENT LYMPH
		(n=2)	(n=2)	(n=5)
CD4+	DQ	17.3±3.0	17.9±3.9	7.8±2.0
TCELLS	DR	19.5±3.6	50.1±2.1	17.9±2.2
CD8+	DQ	12.1±3.3	37.3±17.5	12.4±3.1
TCELLS	DR	17.5±1.3	51.5±1.1	20.5±2.4
T19+	DQ	4.5±3.2	19.3±5.0	4.0±2.0
TCELLS	DR	7.5±5.4	32.1±11.2	12.2±6.0

#### Figure 3.4

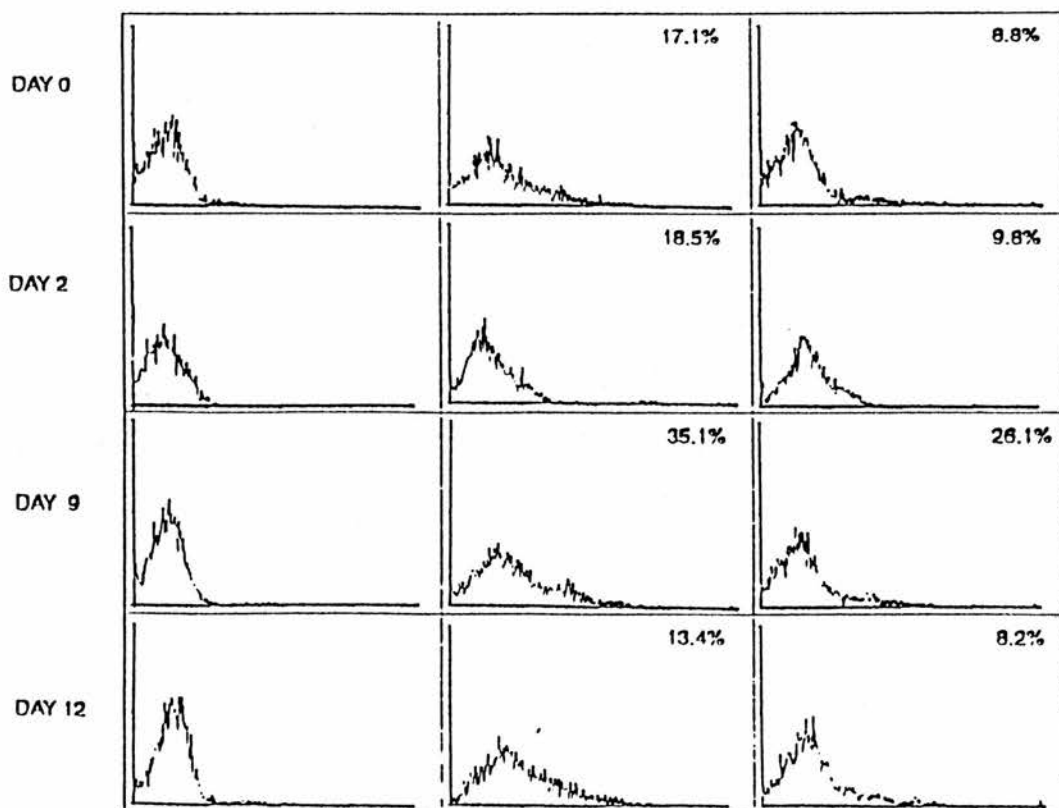
Expression of DR and DQ on T cell subsets following a secondary immune response. Flow cytometry histograms of dual stained efferent lymph cells isolated at daily intervals during the response. Gating on CD4<sup>+</sup> (a, pg 58), CD8<sup>+</sup> (b, pg 58) and T19<sup>+</sup> (c, pg 59) T cells, single histograms were obtained. Each panel shows cells stained with anti-class II DR (VPM54), DQ (VPM36) and the irrelevant antibody negative control (VPM21). The figures represent the percentage positive cells when compared with the irrelevant antibody negative control.

(a) VPM21 VPM54 VPM36



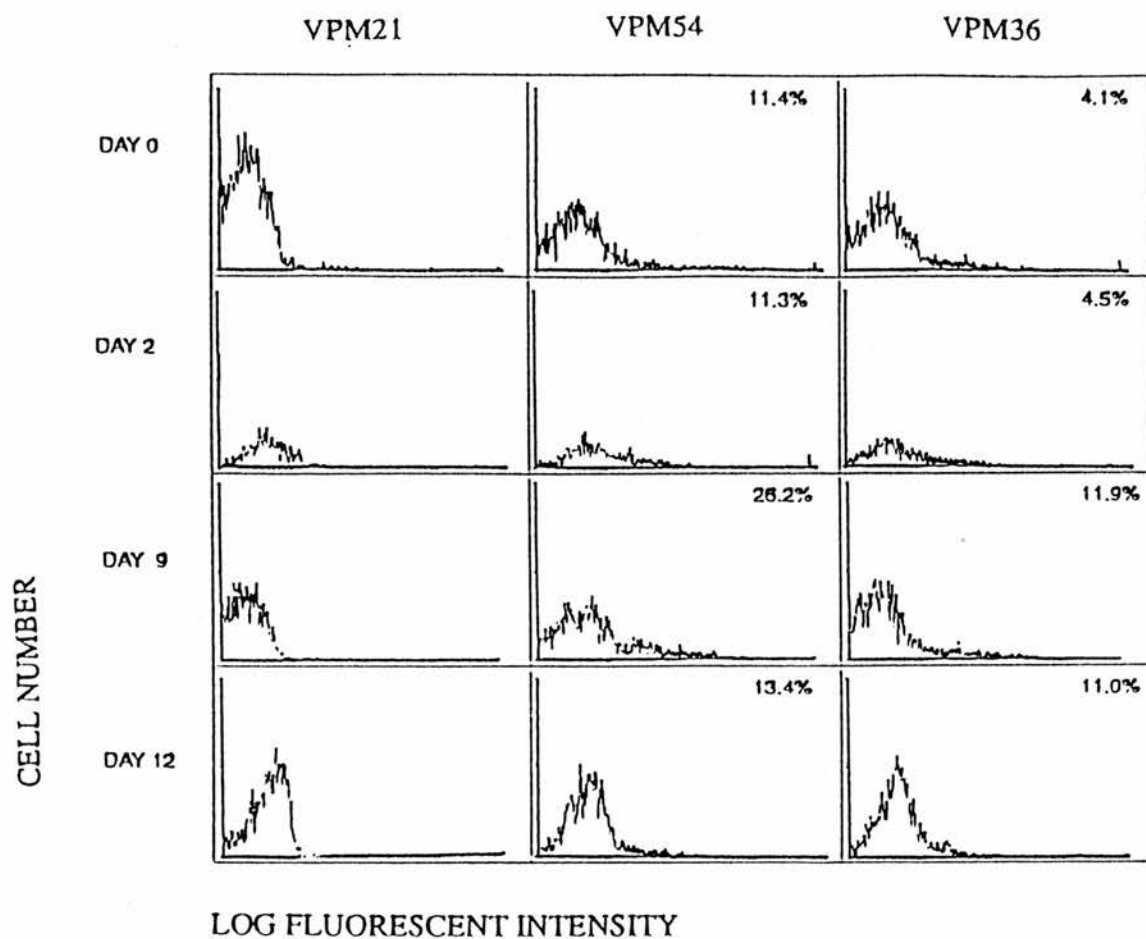
(b)

VPM21 VPM54 VPM36



LOG FLUORESCENT INTENSITY

(c)





Near doubling levels of DR expression occurred on each of the T cell subsets, however much more dramatic levels of increase were observed for DQ expression. Levels of DQ expression trebled on CD4<sup>+</sup> T cells, and doubled on CD8<sup>+</sup> and T19<sup>+</sup> T cells.

### 3.3 Discussion

In this chapter, the characterization of DR and DQ specific mAbs and analysis of antigen expression on resting and activated T cells is described. This data forms an important introduction for the work described in successive chapters.

The characterization of a DR $\alpha$  and a DQ $\alpha$  specific mAbs was accomplished by NH<sub>2</sub>-terminal amino acid sequencing of their antigens. Purification of class II monomers was successfully achieved with the HPEC. Using the HPEC, the individual class II monomers were recovered in fluid phase, transferred onto PVDF membrane and were available for sequencing directly. In comparison, recovery after manual electrophoresis, blotting and staining is typically low and often NH<sub>2</sub>-terminally blocked, making it difficult to obtain sufficient protein for sequencing. The HPEC proved to be a more successful method in purifying proteins for NH<sub>2</sub>-terminal amino acid sequencing.

The amino acid sequence of the class II  $\alpha$  chain antigen recognized by VPM36 in conjunction with 2-dimensional immunoblot analysis (Dutia *et al*, 1993a) confirms that VPM36 recognizes the ovine homologue of DQ $\alpha$ . In humans there are 2 DQA loci one of which is not transcribed. In contrast, the 2DQA loci identified in sheep are both expressed (Scott *et al*, 1991). Studies with two *Ovar*-DQA locus specific probes have revealed extensive polymorphism in both genes (Scott *et al*, 1991; Fabb *et al*, 1993). The presence of the 2 DQA genes has been shown to vary between haplotypes in sheep, a significant proportion of sheep (63/571 tested) do not possess a DQA1 gene (Fabb *et al*, 1993). Whether VPM36 recognizes a single or both DQA gene products is not known. In this study, all the sheep examined expressed the DQA gene products recognized by VPM36. However, in afferent lymph the levels of VPM36 expression in each of the T cell subsets was much lower than that previously reported using the same mAb (Dutia *et al*, 1993a). This may be due to variations in the number of DQA genes possessed by the sheep that are recognized by VPM36. The results shown for VPM36 expression on afferent lymph were derived from two sheep, this is too small a sample size when looking at the expression of polymorphic genes.

A single DRA gene with very few alleles has been identified in sheep (Fabb *et al*,1993). This is consistent with human and murine data. NH<sub>2</sub>-terminal sequence data of the  $\alpha$  chain from VPM54 antigen demonstrates unequivocally that this chain is *Ova*DR-like. Only one difference between the amino acid sequence determined and that predicted from DNA sequence occur in the first 23 amino acids (Fabb *et al*,1993; Ballingall *et al*,1992). This may be a real difference or an error incurred in amino acid sequencing. The residue at this position from the sequence determined from ovine DNA is the same in both human and mouse sequences implying that these are conserved regions. This would indicate that the difference is due to a mistake incurred in amino acid sequencing. However, the published NH<sub>2</sub>-terminal sequence of an  $\alpha$  chain recognized by a different mAb (VPM37) (Dutia *et al*,1993a) has exactly the same difference at this residue which suggest this is real difference.

The level of DR expression reported here is inconsistent with that reported by Dutia *et al* (1993a), when using VPM38 as a DR $\alpha$  chain specific mAb. Much higher levels of staining were observed (30% CD4<sup>+</sup>, 38.4% CD8<sup>+</sup> and 19% T19<sup>+</sup>). Both of these mAbs recognize homologous antigens as determined from NH<sub>2</sub>-terminal amino acid sequence data. That the differences are due to varying antibody binding capacities are precluded as both antibodies show similar intensities of staining on all B cells. Perhaps the differences in levels of expression between both mAbs may be due to differences in background staining.

DQ and DR molecules were found to be expressed on all T cell subsets from afferent lymph, efferent lymph and peripheral blood. Afferent T lymphocytes expressed increased levels of both VPM36 and VPM54 antigens when compared with efferent and peripheral blood T lymphocytes. *In vivo* activation of efferent lymphocytes resulted in increased expression of both DR and DQ molecules, DQ showing a more marked induction on CD4<sup>+</sup> T cells.

The significance of these events is unclear and will be addressed in the adjoining chapters. In afferent lymph both DR and DQ were highly expressed on T lymphocytes when compared with efferent lymph and peripheral blood. Cells circulating in the afferent lymph of sheep have been phenotypically described as memory type (Mackay *et al*,1990). Is class II expression on T cells a marker of memory? Furthermore, upon *in vivo* activation of efferent T cells, both DR and DQ

expression was induced, DQ showing a more marked induction on CD4<sup>+</sup> T cells. Does DQ expression correlate with a recent activation event as has been suggested by Dutia *et al* (1993a)? These questions will be addressed in Chapter 5 by correlating class II expression with other known markers of activation.

In sheep, as in humans, class II molecule expression is associated with activation of the T cell. Activation of the T cell results in the production of cytokines, whose coordinate production regulates the immune response. The pattern of cytokines secreted can subdivide T cells into Th1/Th2 types which have distinct effector functions. To examine whether expression of class II subtypes after activation influences the pattern of cytokines secreted, class II isotype expression will be correlated with cytokine expression. This will be addressed in chapter 6.

## CHAPTER 4 Isolation of T cell subsets.

### 4.1 Introduction

In the previous chapter it was shown that T cell expression of class II increases after *in vivo* activation in antigen primed sheep. The biological significance of this event is not understood but it is possible that class II antigens on T cells are markers of activation status. However, the relationship of class II expression with other known activation markers is largely unknown. By isolating T cell subsets, the expression of class II molecules and activation markers can be analysed on each T cell subset. Two colour flow cytometric staining on the purified T cell subsets would allow the direct correlation of class II isotype expression with a range of available activation markers. This required the evaluation of a suitable cell separation technique. A cell separation technique was also required for the proposed studies on cytokine expression. The isolation of T cell subsets expressing different class II isotypes would allow the correlation of class II isotype expression with cytokine synthesis using molecular biological techniques. Thus the relationship between class II isotype expression and cytokine synthesis could be correlated.

Examples of gross separations of immunological cells include adherence to plastic surfaces which largely removes phagocytic cells and passage down nylon-wool columns which greatly enriches lymphocyte populations for T cells (Julius *et al*, 1973). Using sheep red blood cells, CD2 positive T cells from other species can be separated by rosetting (Shaw *et al*, 1986). The panning technique developed by Mage *et al* (1977) can be used to select cells expressing phenotypic markers by panning on dishes coated with specific mAbs. These methods have various disadvantages including low sensitivity and poor quality of separation. On the other hand, flow cytometry is a powerful method for isolation of subpopulations out of complex cell mixtures. The overwhelming advantage of flow cytometry over other methods is that it is extremely sensitive. However, it is limited by a small separation capacity (approximately  $10^7$  particles per hour). Magnetic beads coated with immunoreactive molecules have emerged as an efficient alternative to flow cytometry. Dynabeads (Dyna, UK) are widely used. More recently, a newer method based on similar principles to Dynabead has been developed, the magnetic activated cell sorter (MACS) (Miltenyi *et al*, 1990).

The most important innovation of the MACS is the ability to couple to the cell

surface biodegradable microbeads which have the size of cellular macromolecules (50-150nm diameter). Cells are sequentially labelled with biotinylated mAbs and streptavidin-conjugated magnetic microbeads. The magnetically stained cells are passed over a ferromagnetic column in the field of a powerful magnet. Labelled cells stick to the magnetized matrix and are separated from the unlabelled cells which flow through. The magnetic fraction can be eluted by removing the column from the magnetic field. This allows both negative and positive separations. The purity of the selection procedures can be assessed by flow cytometric analysis. The advantage of MACS over Dynabeads is that the beads are smaller and biodegradable, plus the presence of microbeads does not affect flow cytometry.

In this chapter, the use of MACS in the selection of T cell subsets is described. T cell populations were both positively and negatively selected and the quality of the separations evaluated. Negative selection identified a novel population of cells and some characterization of these cells was carried out.

## **4.2 Results**

### **4.2.1 MACS - negative selection**

The aim of these experiments was to purify CD4<sup>+</sup> and CD8<sup>+</sup> T cell populations from lymph. Negative selection using the MACS was the method of choice. Through negative selection the selected cells are free of antibody attachments and are thus suitable for dual cytometric analysis to allow direct correlation of class II expression with activation markers on the T cell subsets.

Efferent lymph contains B cells,  $\gamma\delta$  T cells, CD4<sup>+</sup> and CD8<sup>+</sup> T cells. Using biotinylated mAbs reactive with B cells (VPM 30), the T19 molecule of  $\gamma\delta$  T cells (CC15), the CD4<sup>+</sup> T cell subset (SBU-T4) and the CD8<sup>+</sup> T cell subset (SBU-T8), purification of CD8<sup>+</sup> or CD4<sup>+</sup> T cells by negative selection was attempted. The biotinylated mAbs were bound by the streptavidin associated magnetic beads and removed using the MACS. The quality of the selection procedure was then determined by flow cytometry gating on small lymphocytes. Following negative selection for CD4<sup>+</sup> T cells ~73% of the recovered population were of the CD4<sup>+</sup> phenotype. The remainder did not express VPM30 antigen, T19 antigen or SBU-T8 antigen. Likewise for CD8<sup>+</sup> selections ~52% CD8<sup>+</sup> cells were stained. Thus there appeared to be a fifth population of cells present in efferent lymph which did not

express CD4, CD8, T19 and B cell markers. The higher numbers of contaminating cells with CD8<sup>+</sup> selection reflects the higher throughput of cells needed to select out equal numbers of CD8<sup>+</sup> cells. This contaminating population of cells was negative when stained with a general anti-mouse FITC sera, thus excluding the possibility that the magnetic beads were not at saturation level.

#### **4.2.2 Artefacts of MACS selection?**

To discern whether this contaminating population of cells was an artefact of the MACS method the following experiments were conducted.

To confirm that the level of CD4 selection is a real phenomenon and not an artefact of the technique, CD4<sup>+</sup> T cells were pre-labelled with FITC and then negatively selected. Lymph cells were first stained with anti-CD4 conjugated FITC and were then negatively selected using VPM30, SBU-T8 and CC15 mAbs. The negatively selected cells were analysed directly on the FACS. Again the purity of the selection was low (65%) (Fig4.1). As all the FITC stained CD4 cells were recovered in the negative fraction, it is possible that the contaminating cells represent a novel population.

To negate the possibility that antibody capping from the cell surface was occurring, cells were fixed in paraformaldehyde to crosslink surface antigens. Lymph cells were fixed in 0.5% paraformaldehyde and the CD8<sup>+</sup> cells negatively selected. Following separation, the negative population of cells was stained for each of the phenotypic markers CD4, CD8, T19 and B cell. When stained with an anti-CD8, 57% of the selected cells were positive (Fig. 4.2). Thus capping of surface antigens as an explanation was excluded.

There is evidence from the use of Dynabeads that surface antigen detachment can occur during incubation of cells with immunomagnetic beads. On reculturing, antigen depleted cells can recover their normal levels of surface antigen (Rubbi *et al*, 1993). To determine whether the observed contaminating population of cells had their surface antigens detached through the use of magnetic particles, B cells and CD4<sup>+</sup>, CD8<sup>+</sup>, T19<sup>+</sup> T cells were positively removed and the negatively selected cells cultured for 48 hours. Cultured cells were then stained with phenotypic markers. Figure 4.3 shows that re-expression of the CD4, CD8, T19 or B cell antigens did not occur. Unselected efferent cells cultured for 48 hours were used as a control.

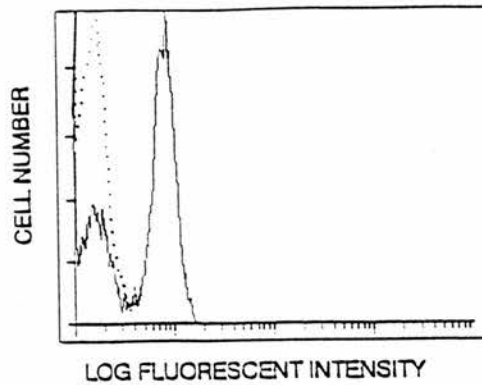


Figure 4.1 Flow cytometric analysis of cells after MACS negative selection for the CD4 antigen. CD4 positive cells were stained with anti-CD4 FITC conjugated antibody prior to separation on the MACS (solid line). Unstained cells are represented by the dotted line.

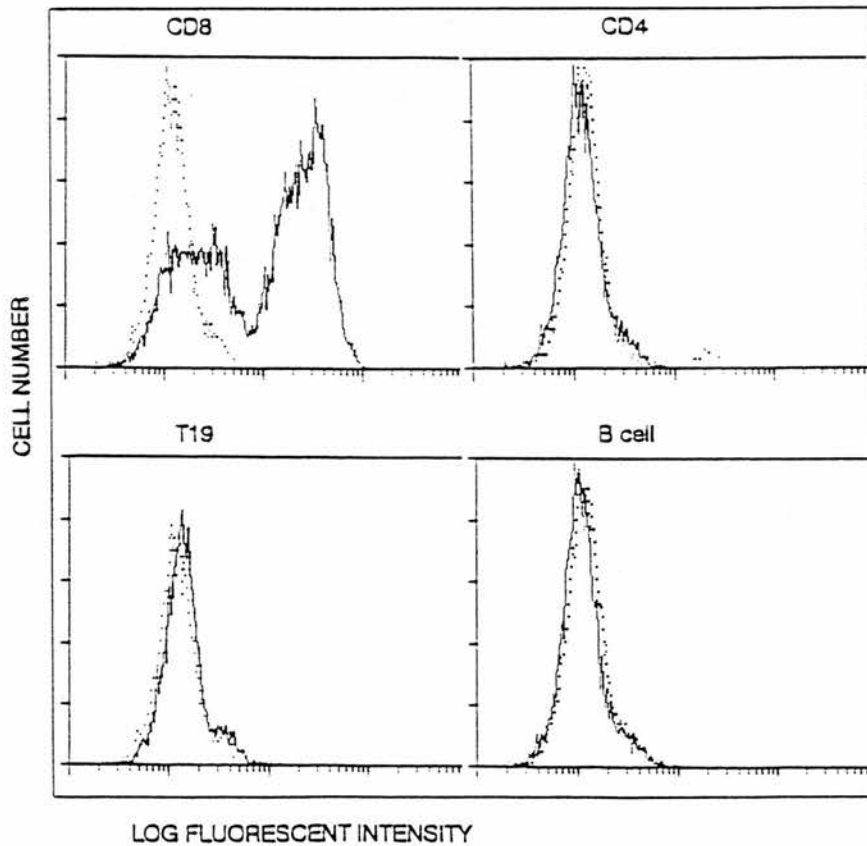


Figure 4.2 Flow cytometric analysis of paraformaldehyde fixed efferent lymphocytes after MACS negative selection for the CD8 antigen. The solid line in each plot represents staining on selected cells with anti-CD8 (SBU-T8), anti-CD4 (SBU-T4), anti-T19 (ST197) and anti-B cell (DU2-104). The dashed line represents staining with NMS a negative control.



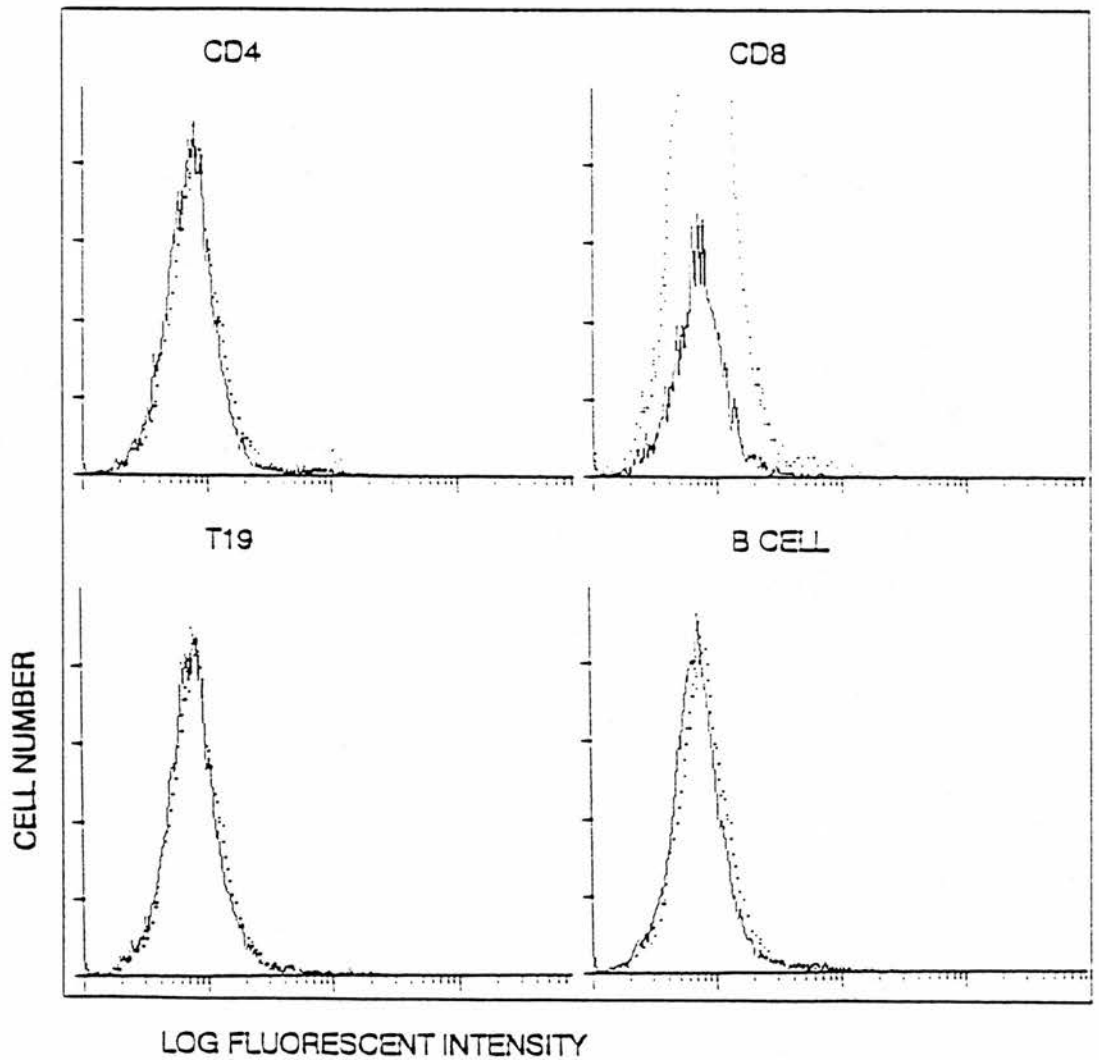


Figure 4.3 Flow cytometric analysis of the null cell population after culturing for 48 hours. Cells were stained with antibodies to CD4 (ST4), CD8 (E95), T19 (CC15) and B cells (VPM30). The dashed line represents staining with VPM21 a negative control.

#### 4.2.3 Null cells

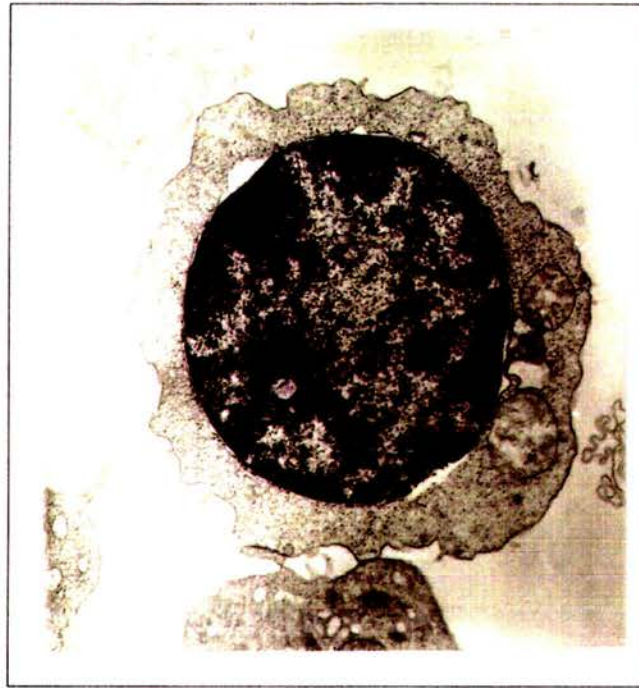
This contaminating population of cells appeared to be a real phenomenon and are henceforth referred to as "null cells". To establish whether null cells are lymphocytes electron microscopy was carried out. Null cells were selected by positively removing CD4<sup>+</sup>, CD8<sup>+</sup>, T19<sup>+</sup> T cells and B cells. Both the negative and positively stained fractions were recovered. Figure 4.4 shows both the positively stained and negatively selected cells. Both cells are typical of small lymphocytes with condensed nuclear chromatin and sparse cytoplasm. The cytoplasm contains few mitochondria and many ribosomes but otherwise few organelles. One can conclude that the null cells are indeed lymphocytes. It was also interesting to see the magnetic beads on the surface of the positively stained cells.

Having established that the null cells are lymphocytes it was decided to stain them with a range of mAbs available in the department. The data is shown in Figure 4.5. In general the null cells express all the markers common to efferent lymphocytes. A notable difference is the absence of CD5 expression. Also a large percentage are LFA1 negative whereas LFA1 positively stains the majority of efferent lymphocytes. The staining pattern of many of these mAbs on CD4<sup>+</sup>, CD8<sup>+</sup> and  $\gamma\delta$  T cells are shown in Figure 5.4 a, b & c in Chapter 5.

There is no available mAb to the  $\alpha\beta$  T cell receptor of sheep. However, there is a hamster anti-human CD3 $\epsilon$  which crossreacts with the ovine homologue. CD3 is associated with both the  $\alpha\beta$  and  $\gamma\delta$  TCR and its presence on null cells would imply the possession of a T cell receptor. The  $\epsilon$  chain of CD3 is cytoplasmic, therefore for analysis of expression, cell lysates were prepared and immunoblotted against HMT3.1. Figure 4.6 shows the CD3 $\epsilon$  chain (20kD) present in both afferent cell lysate (positive control) and the null cell lysates. The presence of CD3 $\epsilon$  indicates that null cells possess a T cell receptor. The ST6 cell line, which does not express CD3 was used as a negative control. The 20,100 marker appears as a diffuse band which would indicate that the markers used were not of optimal quality.

#### NULL CELL

(a)



#### POSITIVELY STAINED CELL

(b)

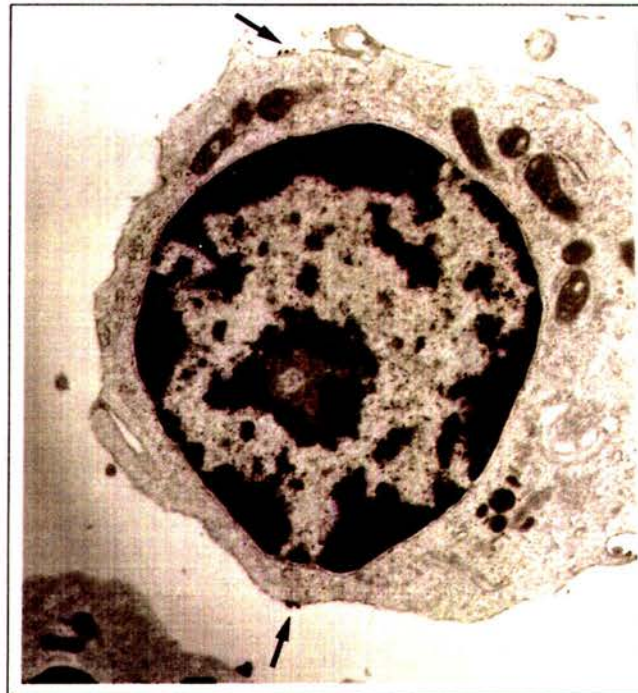


Figure 4.4 Transmission electron micrograph of (a) a null cell, negatively isolated and (b) a positively stained lymphocyte selected by MACS. The arrows indicate the presence of microbeads on the positively selected cell surface.

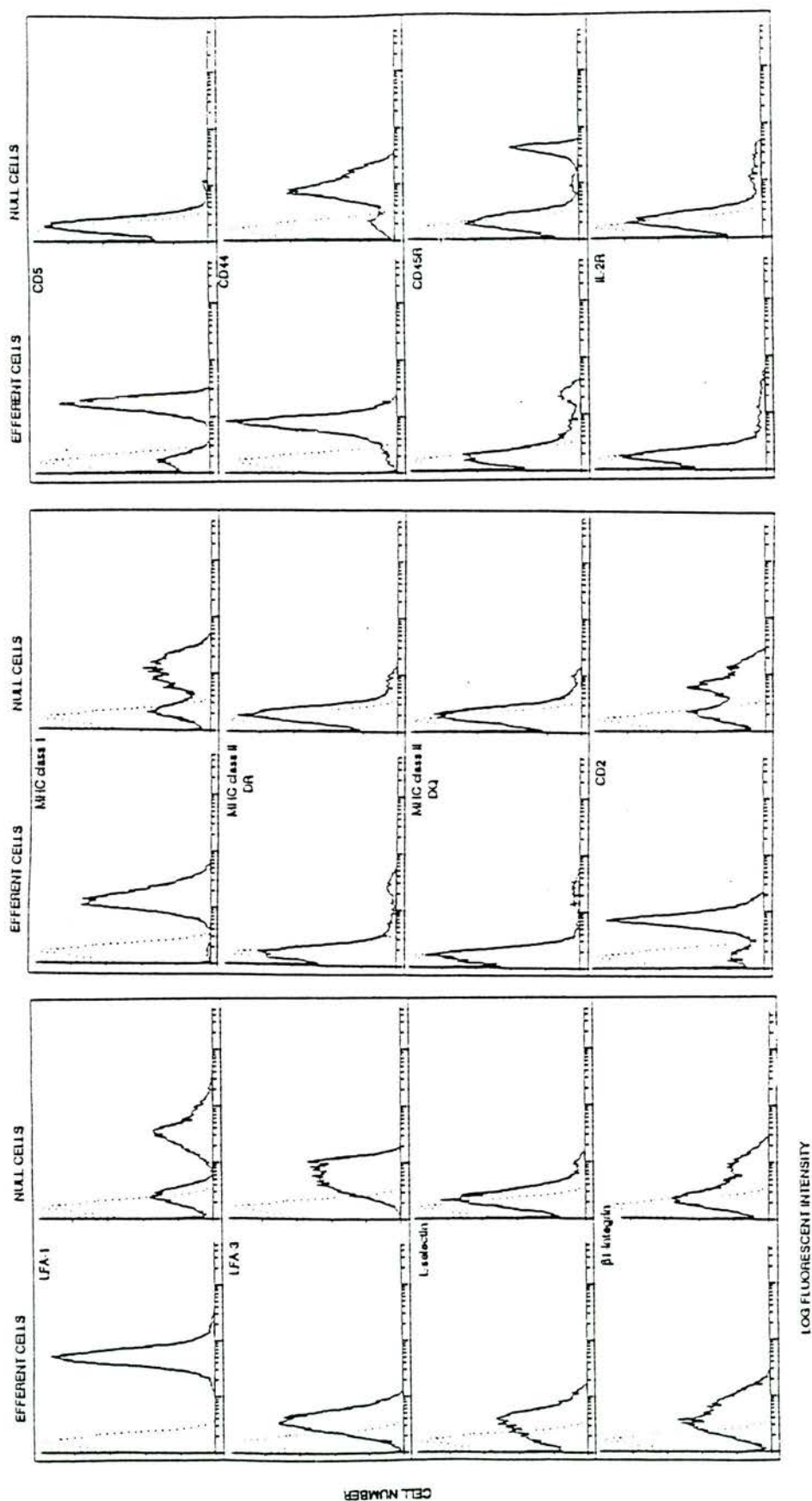


Figure 4.5 Flow cytometric analysis of effluent cells and null cells stained with a range of lymphocyte markers (see Table 2.1 in Chapter 2). VPM21 (dashed lines) was used as a negative control.

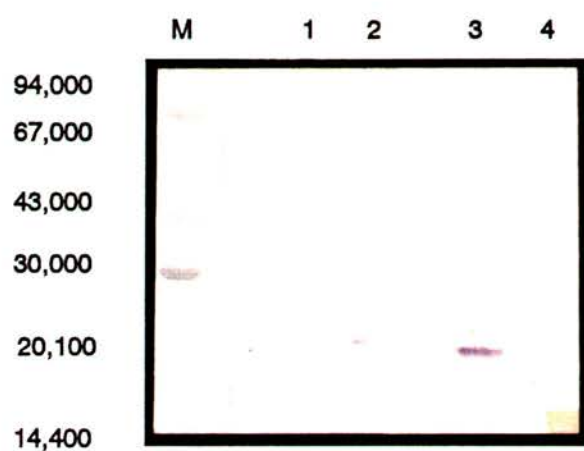


Figure 4.6 Immunoblot analysis of sheep CD3 $\epsilon$  in null cell lysate (lane 1, 2), afferent cell lysate (lane 3) positive control and ST6 cell lysate (lane 4) negative control. Cell lysates were fractionated on 12% SDS-PAGE, transferred to nitrocellulose and probed with HMT3.1 antibody. Molecular weight markers are indicated in the left of the blot.



#### 4.2.4 MACS - positive selection

With the negative selection procedure it was not possible to isolate either CD4<sup>+</sup> or CD8<sup>+</sup> T cells due to the presence of null cells. Because a mAb to a unique marker on null cells was not identified it was not possible to isolate pure populations of CD4 and CD8 positive T cells by negative selection. To circumvent this problem, positive selection of both the CD4<sup>+</sup> and CD8<sup>+</sup> populations of T cells was carried out. With positively selecting cells >95% purity was regularly obtained with CD4<sup>+</sup> selections and >85% purity with CD8<sup>+</sup> selections (Figure 4.7).

Careful selection of mAbs and second layer reagents made it possible to double stain the selected cells. Both CD4<sup>+</sup> and CD8<sup>+</sup> T cells were selected by MACS using biotinylated SBU-T4 and SBU-T8 mAbs which are IgG2a isotype. Green fluorescent staining was achieved using a range of non-IgG2a isotype mAbs followed by isotype specific FITC conjugated antisera. Staining in the red channel was attained using a pan-class II rat derived mAb (SW73.2) detected with a phycoerythrin conjugated anti-rat isotype specific antisera. This allowed class II expression to be correlated with a range of other mAbs directly on the T cell subsets.

Using this procedure, staining patterns of each mAb was established on T cell subsets from the efferent lymph of unstimulated lymph nodes. Because these cells have antibody bound magnetic particles, antibody bound streptavidin and antibody bound phycoerythrin attached to their cell surfaces, it is possible that optimal binding is sterically hindered. Though a certain level of staining for each antibody was detected this may not represent the maximum level of antigen expression. Because T cells show increased expression of class II molecules after *in vivo* activation, it was decided to examine whether the described staining regime would be able to detect the increased levels of class II expression on positively selected CD4<sup>+</sup> cells. The results of this experiment are shown in Figure 4.8. Class II expression on unselected efferent cells is compared with expression on MACS selected CD4<sup>+</sup> cells, on day 8 of an *in vivo* immune response. The efferent cells were stained with anti-class II isotype mAbs (VPM54, VPM36). In parallel, cells were positively selected with the MACS for SBU-T4 and then singly stained with either VPM36 or VPM54 and anti-IgG1 conjugated FITC. The purity of the selection was 97% as determined by staining with an anti-CD4 mAb (ST4).

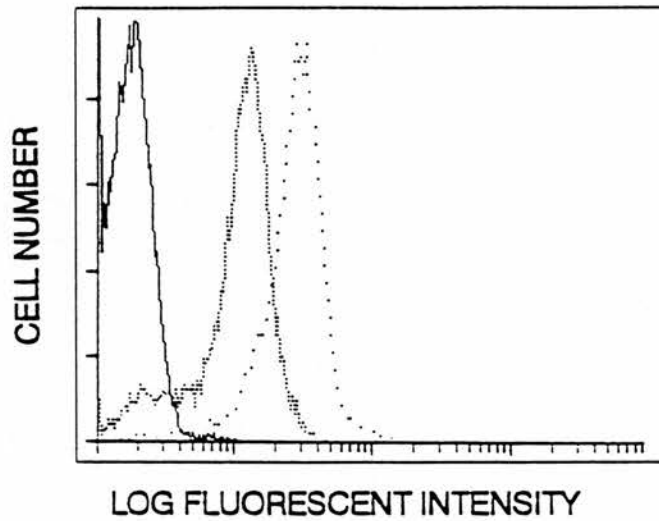


Figure 4.7 Immunofluorescence profile of cells positively selected with the MACS. Cells were stained with both streptavidin-microbeads and streptavidin-phycoerythrin and analysed directly on the FACScan. (·····) represents the CD4 selected cells, (- · - · -) the CD8 selected cells and (—) unstained cells.



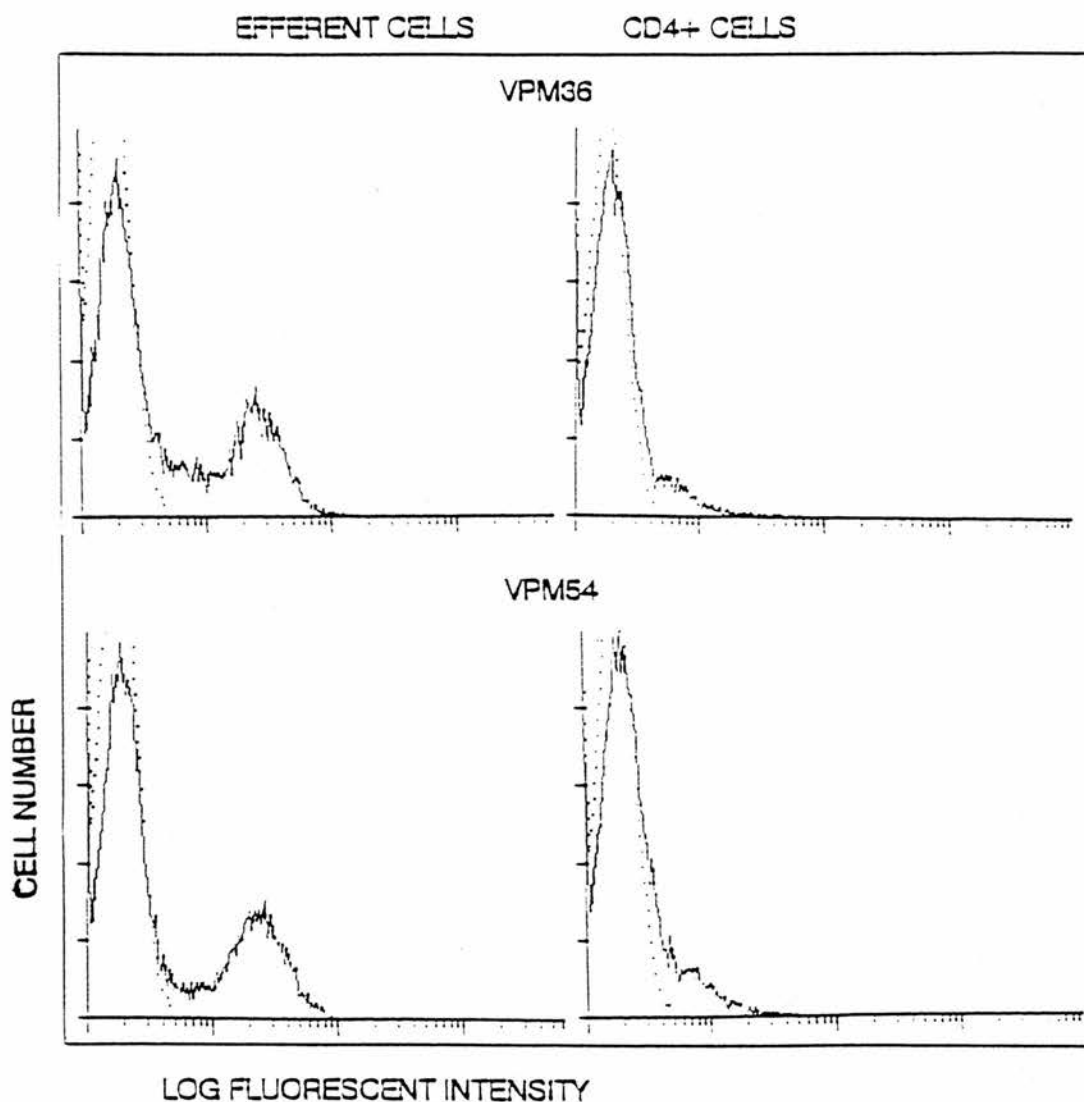


Figure 4.8 Flow cytometric analysis of MHC class II DR (VPM54) and DQ (VPM36) expression on unfractionated efferent cells and positively selected CD4 cells from the same efferent lymph on day 8 of an *in vivo* immune response. The dashed lines represent VPM21 staining (negative control).

The level of staining observed on the unselected cells was not detected on the positively selected and singly stained cells. These cells have many attachments to their surface and steric hindrance effects may have precluded the detection of total surface antigen. Thus use of the MACS in positive selection had to be abandoned.

### 4.3 Discussion

In this chapter the MACS was evaluated for the separation of ovine CD4 and CD8 positive T cell subsets. Purified CD4 and CD8 positive T cells were required for the analysis of activation marker and class II subtype expression by two colour flow cytometric analysis. Both negative and positive selection of cells was undertaken and the quality of the separations analysed by flow cytometric staining.

The presence of a novel cell population precluded the use of a negative selection procedure. The data indicates that the null cells are a novel cell population and not an artefact of the MACS method. From the EM photograph they appear as lymphocytes. Lymphocytes comprise T cells bearing either  $\alpha\beta$  or  $\gamma\delta$  T cell receptors, B cells and Natural Killer cells. The  $\alpha\beta^+$  T cells comprise both CD4<sup>+</sup> and CD8<sup>+</sup> cells for which the null cells were negative. The  $\gamma\delta$  T cells of sheep bear the T19 molecule (WC1) which the null cells did not express. Null cells were also negative for B cell markers. The other cell type that constitute lymphocytes is the Natural Killer (NK) cell. NK cells have a large granular morphology and lack CD3 $\epsilon$  expression (Lanier *et al*, 1986), this precludes null cells as null cells expressed CD3 $\epsilon$  and had the morphology of small lymphocytes. A mAb to a unique marker on null cells would help to phenotype and quantify this population.

From FACS analysis null cells express similar markers to other lymphocytes when analysed within the small lymphocyte gate. The notable difference being the lack of CD5 expression. The absence of CD5 is interesting as CD5 is expressed by all sheep T cells, thymocytes and a proportion of B cells (Mackay, 1988) only on T cell activation is CD5 expression lost (Hopkins and Dutia, 1990). The absence of CD5 expression, could perhaps imply that the null cells are activated T cells, however the null cells did not have the morphology of activated T cells. Because CD5 is expressed on all T cells and thymocytes in sheep, the lack of expression on null cells may indicate a different ontogeny for these cells. Indeed, CD4<sup>-</sup> CD8<sup>-</sup>  $\alpha\beta^+$  T cells have been identified in both mice and humans which do

not appear to undergo classical positive or negative selection in the thymus and it has been suggested that they develop extrathymically (Andreu-Sanchez *et al*, 1991). In healthy humans the CD4<sup>+</sup> CD8<sup>-</sup> T cells represent small populations of both PBL and cells of the thymus (Brooks *et al*, 1993). Functionally, these cells in mice have been shown to recognize bacteria (Abo *et al*, 1991) while in humans they recognize CD1 molecules in association with bacterial products (Porcelli *et al*, 1992). The null cell population identified may represent the  $\alpha\beta^+$  CD4<sup>+</sup> CD8<sup>-</sup> T cells of mice and humans. Further work is required to fully characterize these cells and elucidate their functional role.

Using a positive selection procedure highly purified cells were separated. However the positively stained cells could not be further stained for flow cytometric analysis. As the positively selected cells are bound by magnetic particles, these molecules may sterically hinder further binding to the cell surface. Because the cells were fixed in paraformaldehyde prior to purification, this effect is not due to capping of the antigens. Fluorescent labels are smaller than the magnetic particles used for MACS which would explain why triple and double staining can be achieved for flow cytometric analysis. There have been many reports on the use of MACS for cell separations. However these generally have involved the positive selection of cells for *in vitro* cell culture where the presence of beads has been found not to affect functional assays (Abts *et al*, 1989; Jacobs *et al*, 1993).

Though the MACS could not be used for these studies it did prove to be an efficient method for isolating cells. When negative selection was employed the removal of the positively stained cells was almost complete. It was the presence of a novel cell population that precluded the use of a negative selection procedure. The positive selection procedure also yielded highly purified cell fractions. Positively selected cells were unsuitable, as the magnetic particles appeared to affect two colour flow cytometric analysis. Thus in conclusion, the MACS had limited capabilities for the required experiments.

## **CHAPTER 5 The correlation of class II expression with activation/adhesion molecule expression on T cell subsets.**

### **5.1 Introduction**

Following *in vivo* antigenic stimulation, T cells show increased expression of both DR and DQ class II isotypes. It has been proposed that the pattern of MHC class II expression may reflect different levels of T cell differentiation and activation (Dutia *et al*,1993a), the DQ<sup>-</sup>DR<sup>-</sup> phenotype changing to the DQ<sup>+</sup>DR<sup>+</sup> phenotype on activation and the DQ<sup>-</sup>DR<sup>+</sup> T cells representing resting recirculating memory T cells. To examine this proposition the expression of activation/adhesion molecules and class II subtypes on T cell subsets was studied. Originally it was hoped to correlate class II expression directly with expression of activation markers on isolated T cell subsets, however from results described in the previous chapter this proved problematic. As a consequence the correlation of class II expression with activation/adhesion marker expression was analysed indirectly by two colour flow cytometry. Cells were stained for cytometric analysis in one channel with phenotypic markers (CD4, CD8 and T19) and with activation/adhesion markers and class II in the second channel.

Secondary antigen challenge mediates a vigorous immune response from immunologically experienced cells. This enhanced response is due to the persistence in the host of antigen specific cells and their increased frequency on stimulation. These memory T cells also exhibit different circulatory behaviour to virgin cells and the selective expression of an array of adhesion molecules facilitates their extravasation into specific tissues. Several markers that distinguish activation states of T cells have been identified. T cells which have not encountered antigen express high molecular weight isoforms of CD45 (CD45RA/B/C), a low density of both CD44 and  $\beta$ 1-integrin and a high density of L-selectin. The reciprocal phenotype is displayed by memory/effector T cells primed to various environmental antigens (CD45RA<sup>lo</sup>, CD44<sup>hi</sup> and L-selectin<sup>lo</sup>) (Sanders *et al*,1988; Mackay *et al*,1990). In addition, memory phenotype T cells express higher levels of several adhesion molecules such as ICAM-1, LFA-1, CD2, LFA-3 and  $\alpha$ 4 $\beta$ 1 integrin (reviewed in Mackay,1993). In this study class II isotype expression is correlated with the expression of CD45RA, CD44, CD2, LFA-3, L-selectin and the  $\alpha$ 4-heterodimers.

CD45 is a transmembrane glycoprotein whose cytoplasmic domain shows protein tyrosine phosphatase activity (Tonks *et al*,1988). CD45 isoform expression subdivides T cells into reciprocal subsets expressing either high molecular weight isoform (CD45RA/B/C) or the low molecular weight isoform (CD45RO). The CD45RO subset responds *in vitro* to recall antigens and provides help for antibody synthesis (Morimoto *et al*,1985). The similarities between species led to the widespread acceptance of high and low molecular weight isoforms as markers of naive and memory T cells respectively (Mackay,1991). It is now clear that this distinction is not absolute as reversal of isoform expression on previously primed cells occurs (Sparshott and Bell,1994). CD45 isoforms have been shown to regulate signal transduction differentially through the TCR complex. The high molecular weight isoform enhancing TCR signalling while CD45RO requires a costimulus to augment cellular proliferation (Chui *et al*,1994). Further evidence from CD45 deficient T cell lines has shown that CD45 expression is an absolute requirement for TCR signalling (Trowbridge and Thomas,1994). CD45 isoform expression may be more appropriately described as a marker of activation state.

CD44 was one of the first molecules employed to distinguish memory phenotype T cells. Murine T cells can be subdivided into CD44<sup>hi</sup> and CD44<sup>lo</sup> subsets, the CD44<sup>hi</sup> T cells representing antigen activated cells (Cerottini and MacDonald,1989). In sheep, CD44<sup>lo</sup> T cells in lymphatic vessels are resting cells whereas the CD44<sup>hi</sup> cells are effector cells dividing rapidly over a period of days (Mackay *et al*,1990). CD44 interacts with several cell surface and matrix components, including fibronectin, collagen and hyaluronate (Miyake *et al*,1990). There is no direct evidence that CD44 binds to the high endothelial venules or activated endothelium, instead it has been proposed that CD44 functions in activating other adhesion molecules and the modulation of CD2 has been found (Herrlich *et al*,1993).

CD2 is a member of the immunoglobulin superfamily expressed on CD4 and CD8 T cells. Its counter receptor is LFA-3, which is also a member of the immunoglobulin super family. The driving force in regulation of cell-cell adhesion mediated by the CD2/LFA-3 mechanism is T lymphocyte activation. Close cell contact between circulating cells is opposed by the negative charge of sialic acid. This repulsive negative charge is reduced on activated T cells. The differences in

surface charge contributes to different levels of adhesion mediated by CD2/LFA-3 (reviewed in Springer,1990). The CD2/LFA-3 interaction also induces signal transduction events involved in the regulation of T cell responses. CD2 mediated activation of human T cells has been shown to induce class II expression (Oshima and Eckels,1990b). After *in vitro* activation of naive human T cells increased expression of CD2 and LFA-3 has been reported (Sanders *et al*,1988). Similarly, it has been found that *in vivo* activated sheep T cells show increased expression of CD2 and LFA-3 (Mackay *et al*,1990).

L-selectin was the first identified mediator of lymphocyte homing to peripheral lymph node HEV (Gallatin *et al*,1983). Naive T cells are L-selectin positive enabling them to enter the lymph node directly from the blood while memory T cells lack this receptor and reach the lymph node via afferent lymph draining the tissues. Following activation the down-regulation of L-selectin and up-regulation of other adhesion molecules on memory T cells suggest that they are less apt to bind to lymph node HEV but more prone to bind to other endothelial surfaces (Mackay,1991).

The mAb 218 recognizes  $\alpha 4$  integrins of which 2 distinct  $\alpha 4$ -heterodimers have been identified  $\alpha 4\beta 1$ (VLA-4) and  $\alpha 4\beta 7$  (Holzmann and Weissman, 1989). Memory cells in humans and mice which show a trophism for the gut express the  $\alpha 4\beta 7$  integrin, while  $\alpha 4\beta 1$  is one of the principal adhesion molecules for lymphocyte extravasation to inflammatory sites (van Kooyk and Figdor,1993).

Because the frequency of antigen specific lymphocytes is low, continuous migration through most tissues of the body enhances the likelihood of cells of appropriate specificity coming into contact with their individual antigen. It has been proposed that to optimize their efficiency, naive and memory T cells have different circulatory routes (Mackay,1991). Afferent lymph contains mainly cells of the memory phenotype while efferent cells contain a mixture of naive and memory phenotypes (Mackay *et al*,1990). To establish staining patterns for each of the described activation/adhesion markers, staining on both afferent and efferent lymph cells was established. The staining pattern following activation in the efferent lymph was then correlated with class II isotype expression. Proliferation assays and assays for specific antibody (gel diffusion) were carried out to establish that the animals were adequately primed to antigen.



## **5.2 Results**

### **5.2.1 Proliferation and gel diffusion assays.**

To establish that the sheep used were responding to antigen, proliferation assays were conducted on their PBLs. Gel diffusion assays were also conducted to measure levels of ovalbumin specific antibodies. These results are shown in Figure 5.1. A stimulation index of  $>6$  and an antibody equivalence of  $\sim 30 \mu\text{g/ml}$  ovalbumin was required before the typical kinetic changes that characterize a secondary immune response were detected. In animals primed with ovalbumin with low antibody responses and low stimulation index from proliferation assays there were no observed changes in cell kinetics after antigen challenge to the cannulated node. This is similar to what has been found in primary immune responses of the sheep (Hopkins *et al*, 1993). Sheep were repeatedly stimulated with ovalbumin until an adequate antibody response was detected. A threshold level of antigen priming appears to be required.

### **5.2.2 Changes in efferent lymph following antigen challenge**

Antigen challenge to a lymph node leads to pronounced changes in the output of cells (Hall and Morris, 1965). Secondary immune responses were characterized by cell shutdown within the first 24 hours followed by an increase in lymphocyte output between 50 and 150 hours (Fig 5.2a). By 9/10 days after antigen challenge cell output and lymph flow had returned to resting levels. Changes in the percentage of  $\text{CD4}^+$ ,  $\text{CD8}^+$  and  $\gamma\delta$  T cells following antigen challenge are shown in Figure 5.2b. The percentage of  $\text{CD4}^+$  cells increases on days 1, 2 and 3 post antigen challenge while the percentage of  $\text{CD8}^+$  cells increases on days 2 to 5.  $\gamma\delta$  T cell levels remained constant.



Sheep No.	Antigen	Stimulation Index
BT169	OVA	7.3
	HSA	0.8
	ConA	20
BT170	OVA	7.4
	HSA	1.5
	ConA	14.2

Table 5.1  
Proliferative response of PBMC to ovalbumin, human serum albumin and Con A from two sheep primed to ovalbumin. The proliferative response is presented as stimulation indices calculated as follows  

$$SI = \frac{\text{mean proliferative response with antigen}}{\text{mean proliferative response without antigen}}$$

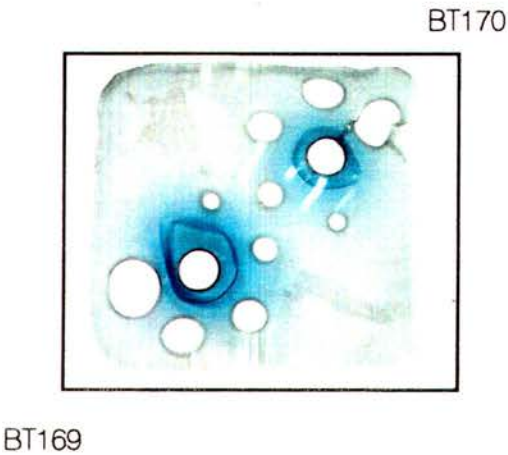
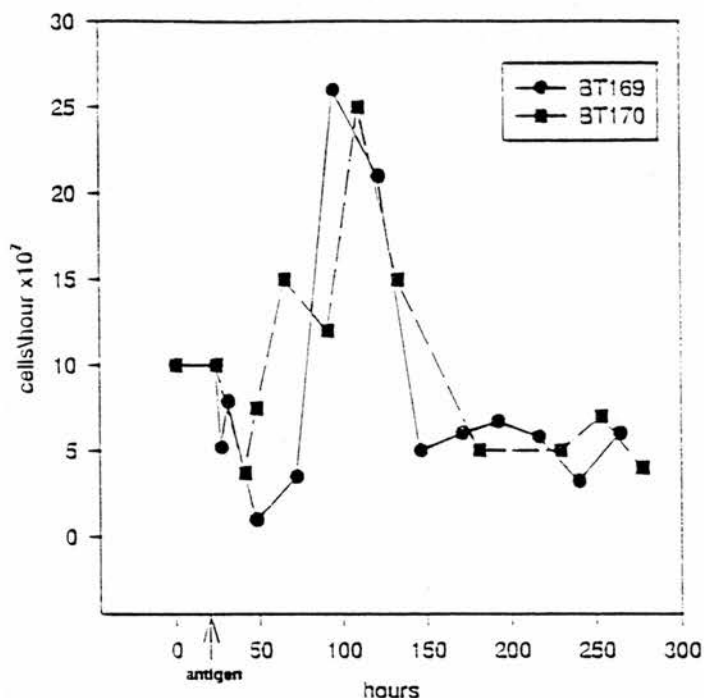
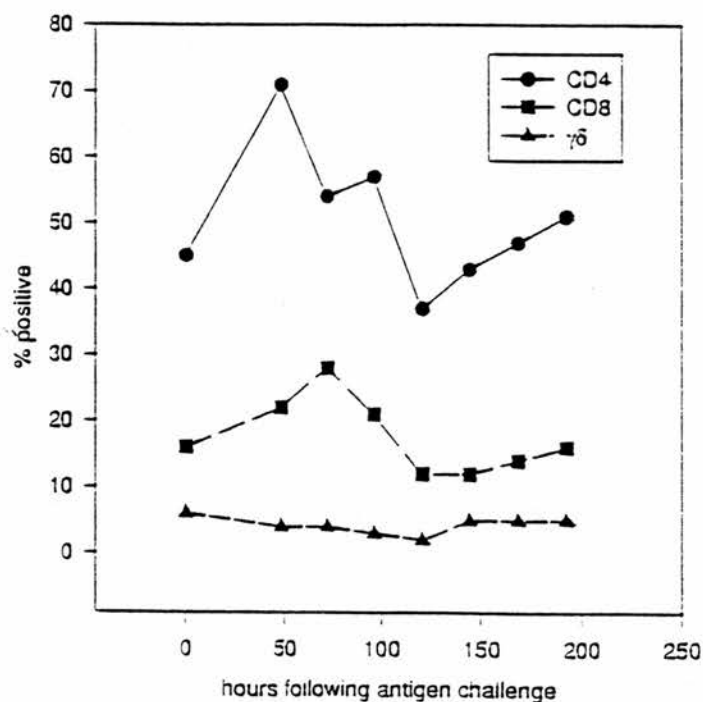


Figure 5.1  
Gel diffusions assays with sera from the same sheep as in Table 5.1. Ovalbumin at a concentration of 0.5mg/ml was titrated in the outer wells and serum added to the inner well.

Figure 5.2



(a) Changes in total cell output from two sheep during a secondary immune response *in vivo*.



(b) Changes in the percentage staining of CD4<sup>+</sup>, CD8<sup>+</sup> and T19<sup>+</sup> efferent cells following *in vivo* antigen challenge. Cells were collected from a cannulated lymph node before and after antigen challenge, stained with SBU-T4, SBU-T8 and CC15 and analysed by flow cytometry.

### 5.2.3 Afferent and efferent staining for activation/adhesion markers

Cells of the afferent lymph are described as memory type while in efferent lymph they are mostly of the naive type (Mackay *et al*,1990). Using monoclonal antibodies to activation/adhesion molecules the staining patterns on both afferent and efferent lymph was established to distinguish between naive and memory patterns of staining (Fig 5.3). The CD4<sup>+</sup> cells of efferent lymph expressed lower levels of  $\alpha$ 4 integrin when compared with afferent CD4<sup>+</sup> cells.  $\alpha$ 4 integrin was expressed on a higher percentage of afferent CD4<sup>+</sup> cells and showed a higher intensity of staining as evident from the peak channel data. L-selectin expression showed a reciprocal pattern, decreased expression was observed on afferent cells. Expression of CD45R was absent on afferent cells whereas 67% of efferent cells expressed CD45R. This data is consistent with the reported naive and memory phenotypes of efferent and afferent lymph cells respectively. Differences in CD2, CD44 and LFA-3 expression levels are not as obvious. Slight variations in levels of intensity were observed.

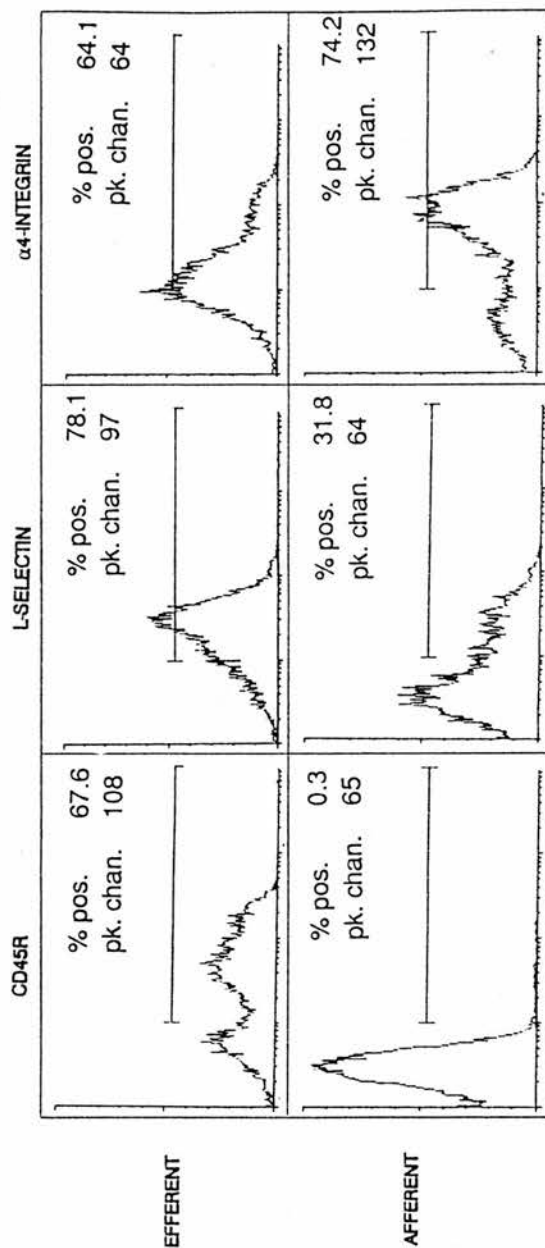
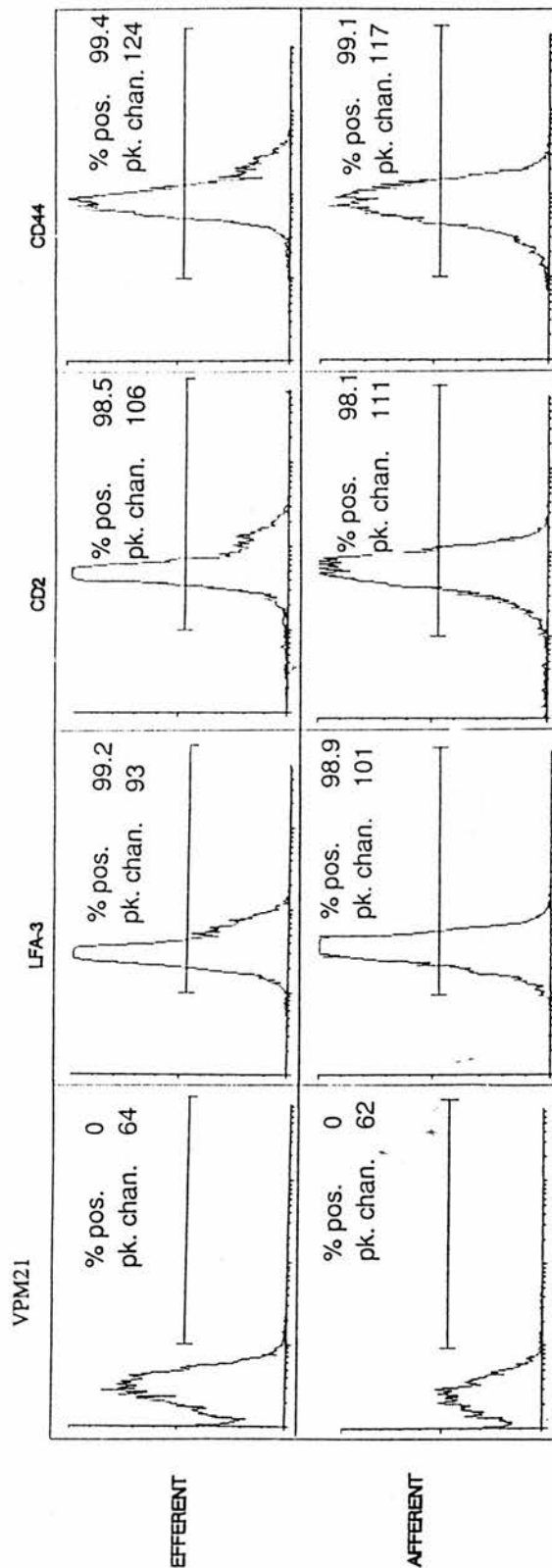
The CD8<sup>+</sup> T cells were also divided into naive and memory phenotypes. L-selectin was expressed on 30% afferent CD8<sup>+</sup> cells and 93% of efferent CD8<sup>+</sup> cells.  $\alpha$ 4 integrin was expressed by comparable numbers of CD8<sup>+</sup> cells in efferent and afferent lymph, however the intensity of staining was higher on afferent cells. Levels of CD45R expression were negligible on afferent CD8<sup>+</sup> cells while 87% of efferent cells were positive. These differences subdivide CD8<sup>+</sup> T cells into afferent memory and efferent naive types. Again differences in expression of CD2, LFA-3 and CD44 were less clear. High levels of CD2<sup>-</sup> CD8<sup>+</sup> cells were observed on both efferent and afferent lymph. The CD8 molecule is expressed on a proportion of NK cells and  $\gamma\delta$  T cells (Mackay *et al*,1990) and because  $\gamma\delta$  T cells do not express the CD2 molecule, this may account for the high percentage of CD2<sup>-</sup> CD8<sup>+</sup> cells.

The anti-CD45 mAb (73B) used in these studies has been shown to recognize a high molecular weight isoform, possibly CD45RA. This antibody stains CD4 and CD8 positive T cells, while staining on  $\gamma\delta$  (T19<sup>+</sup>) T cells has been shown to be less than 10% (Dutia *et al*,1993b). The data presented confirms this finding for  $\gamma\delta$  T cells. CD2 is not expressed on  $\gamma\delta$  T cells (Mackay *et al*,1988) and the data shows a negative staining pattern for CD2.

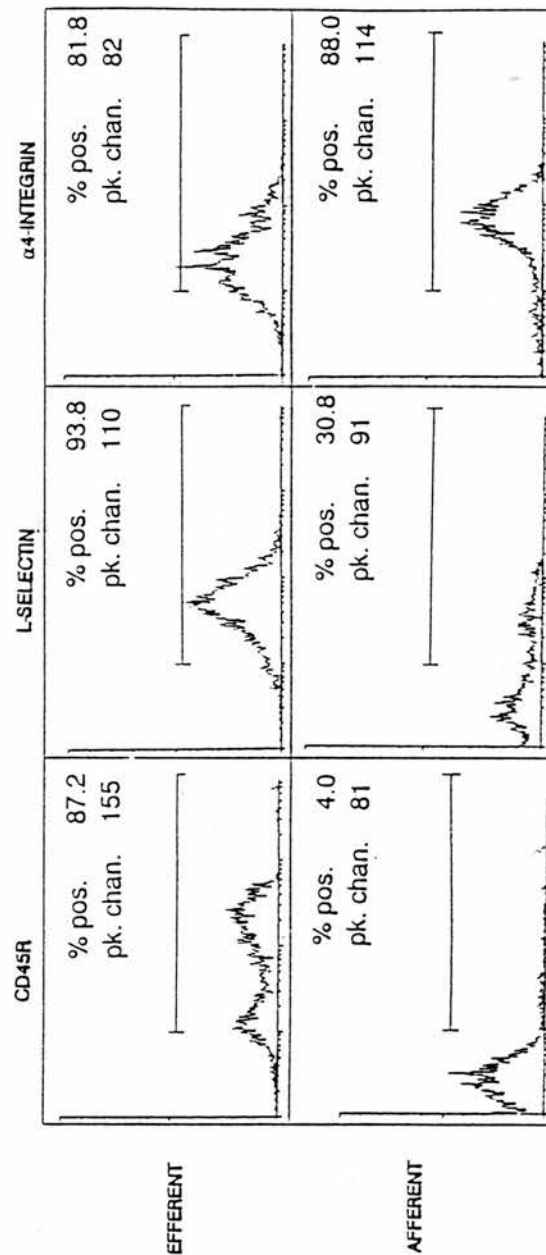
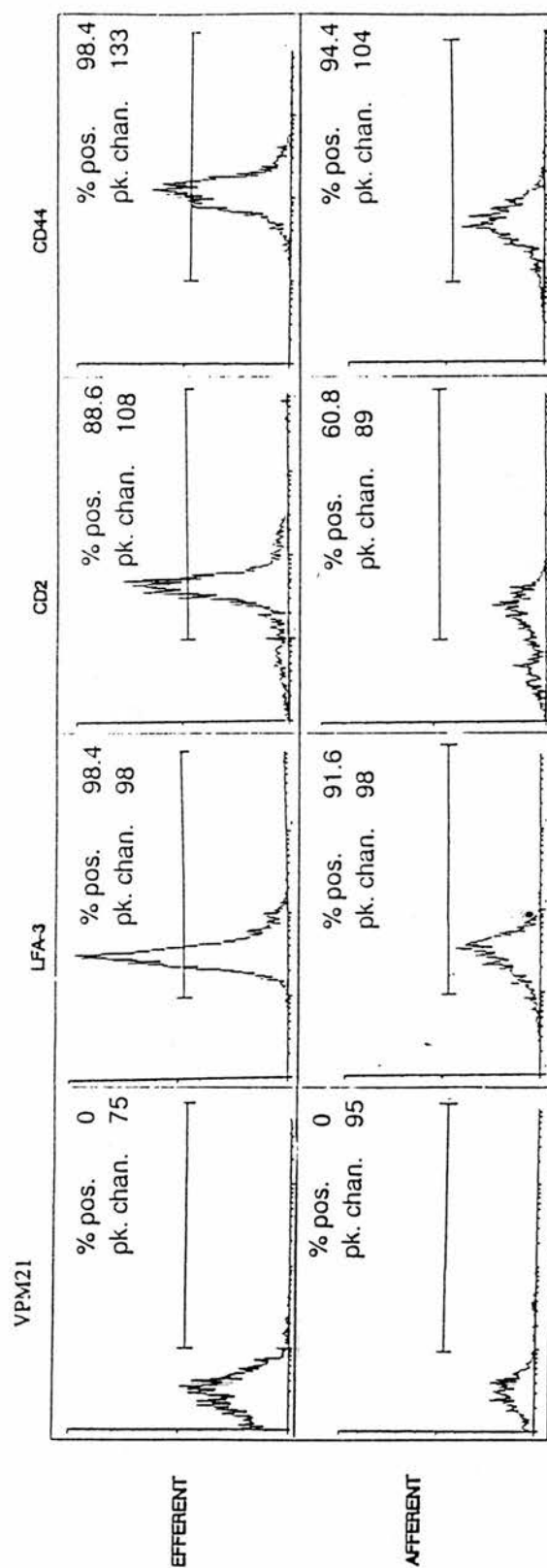
### Figure 5.3

Flow cytometry histograms of double stained efferent cells compared with double stained afferent lymph cells. Adhesion/activation marker expression (FL1) is displayed as single histograms gated on (A) CD4<sup>+</sup>, (B) CD8<sup>+</sup> and (C) T19<sup>+</sup> T cells (FL2). Gates were set on the negative control (VPM21) profile for each of the T cell subsets. The figures represent the percentage positive cells (% pos.) when compared with the negative control. Peak channel (pk. chan.) data is also shown. Cells were derived from unstimulated efferent (sheep BT169) and afferent (sheep BT165) nodes.

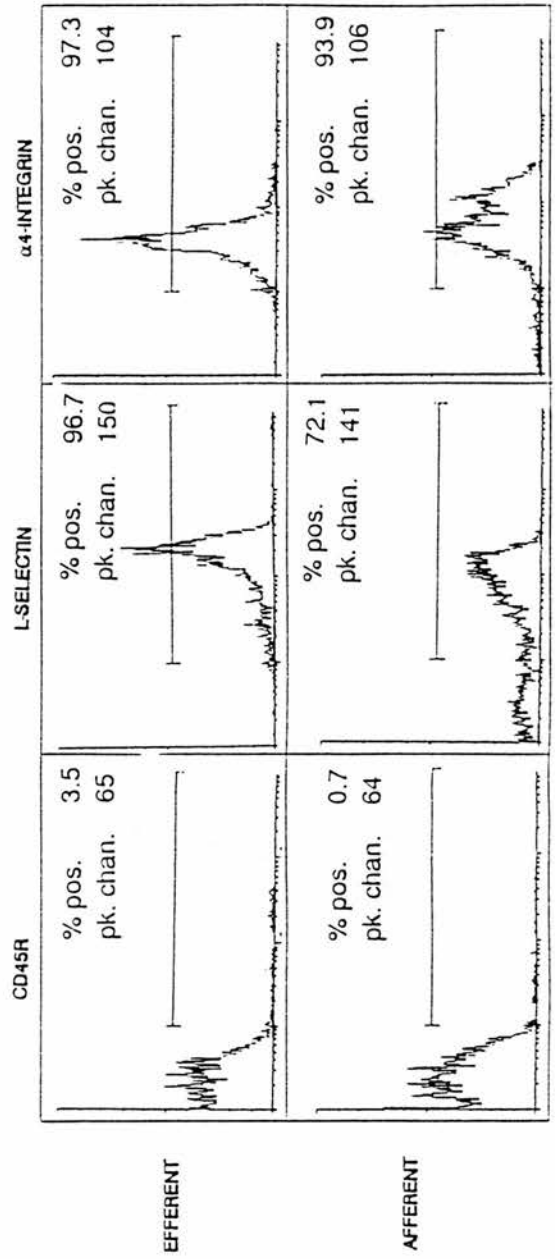
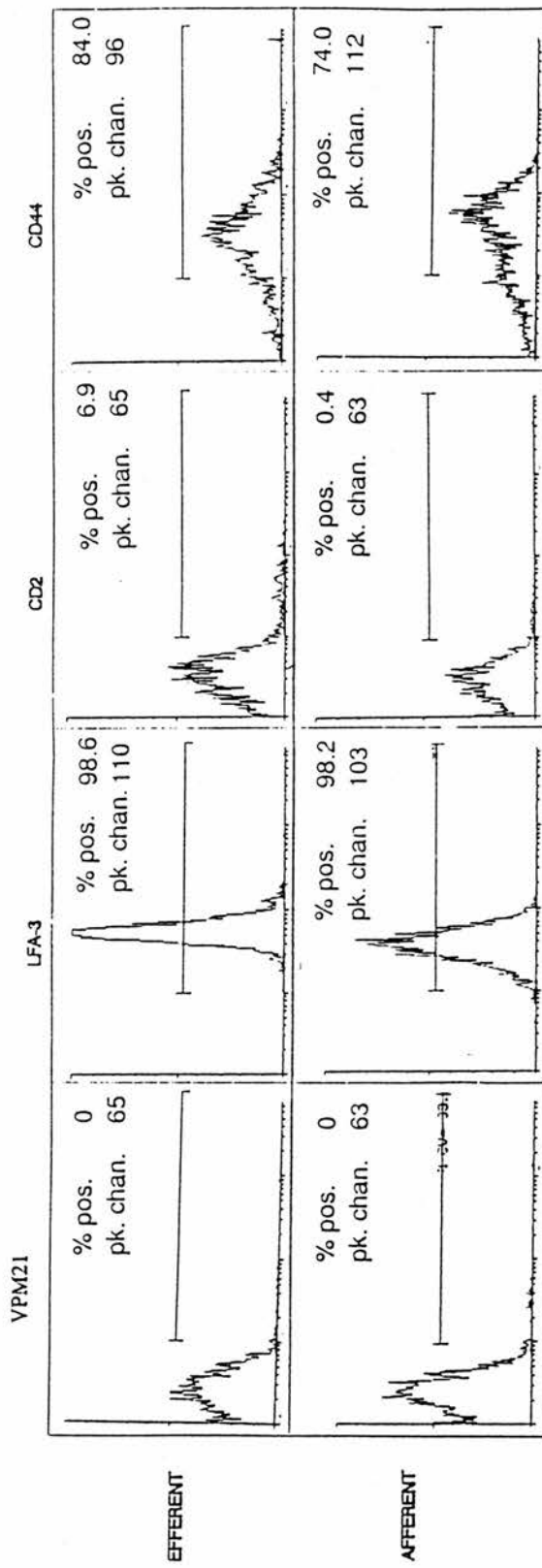
A



B



C





Both efferent and afferent  $\gamma\delta$  T cells express L-selectin at a greater intensity to CD4<sup>+</sup> and CD8<sup>+</sup> cells. The peak channel data for L-selectin expression on  $\gamma\delta$  T cells reaches 150 compared with 110 for CD8<sup>+</sup> cells and 97 for CD4<sup>+</sup> cells. Differences in L-selectin,  $\alpha 4$  integrin, LFA-3 and CD44 expression between efferent and afferent lymph were difficult to discern.  $\gamma\delta$  T cells in both efferent and afferent lymph appear to express a memory phenotype based on expression of both CD45R and  $\alpha 4$  integrin.

Overall, CD4 and CD8 positive T cell subsets in afferent lymph expressed a predominantly memory phenotype while efferent lymph T cells were mainly of the naive phenotype. Differences between efferent and afferent  $\gamma\delta$  T cells were not similarly marked. For comparison class II DR and DQ expression on efferent and afferent lymph T cell subsets was shown in Figure 3.3 a, b and c. The DR<sup>+</sup>DQ<sup>+</sup> phenotype of afferent CD4<sup>+</sup> and CD8<sup>+</sup> T cells correlating with a memory phenotype and the DR<sup>-</sup>DQ<sup>-</sup> of efferent T cells correlating with a naive phenotype. The DR<sup>+</sup>DQ<sup>-</sup> T cells in efferent lymph may represent a low incidence of memory phenotype cells.

#### **5.2.4 Expression of adhesion/activation markers on T cell subsets following *in vivo* stimulation.**

Having established the staining patterns for memory and naive cells, these mAbs were used to follow the events of cellular activation. Antigen was delivered to the draining area of the cannulated node of antigen primed sheep. Cells were analysed by flow cytometry gating on small lymphocytes. 10,000 events were counted for each sample. The proportions of T cell subsets varied during the immune response and this is reflected in the numbers analysed. For example, when the numbers of CD4<sup>+</sup> T cells were high the proportion of  $\gamma\delta$  T cells analysed was reduced. Two sheep were used in this study. Analysis at 4 time points was achieved for sheep BT169, while analysis at 2 time points was obtained for sheep BT170. The results are presented for each of the T cell subsets in Fig 5.4 a,b & c and Fig 5.5 a,b & c respectively. Class II DR and DQ staining on sheep BT169 was shown in Fig 3.4 a, b and c. Class II DR and DQ staining on BT170 T cells is included in Fig 5.5.

##### **5.2.4.1 CD4<sup>+</sup> T cells**

During the secondary immune response of sheep BT169, L-selectin expression showed a bimodal distribution pattern on days 2 and 9, though the

percentage positive cells increased on day 2 numbers fell below day 0 values by day 9. Resting levels were restored on day 12. The loss in L-selectin staining intensity was not as dramatic as that seen in afferent lymph. An increased percentage of CD4<sup>+</sup> cells stained with mAb 218 ( $\alpha$ 4-heterodimers) was observed on day 2. A variable pattern of CD45R staining was observed throughout the time course. Expression of CD2 on days 2 and 9 appeared to show a marginal increase in intensity, this was not reflected by the peak channel data. No variations in LFA-3 and CD44 were observed.

On the second sheep examined, expression of CD2 and CD44 by day 4 was increased. L-selectin expression was reduced from 78% on day 0 to 50% on day 4. CD45R expression was also reduced, 26% on day 0 to 9% on day 4. Though the percentage  $\alpha$ 4 integrin positive cells remained the same on days 0 and 4, the cells collected on day 4 expressed a higher intensity of staining as evident from the peak channel data (Fig5.5A). From the results of these two sheep, it can be concluded that CD4<sup>+</sup> T cells in efferent lymph show an increased expression of the memory phenotype on day2/4.

Levels of class II expression on CD4<sup>+</sup> T cells also increased over the time course for both sheep. Sheep BT169 showed increased expression of both DR and DQ by day 2 with near doubling levels expressed on day 9 (Fig3.4a). By day 12 class II expression had returned to resting levels.

#### **5.2.4.2 CD8<sup>+</sup> T cells**

The staining pattern on day 2 for CD8 cells was marked by a higher intensity of CD2 and LFA-3 expression which is reflected in the peak channel data. No significant changes in CD44 expression occurred (Fig 5.4B). Loss of the CD2<sup>+</sup> population on day 2 was coincident with the reduced numbers of  $\gamma\delta$  T cells analysed per sample. No significant difference in L-selectin expression occurred between day 0 and 2 however the percentage L-selectin positive cells decreased on day 9 and 12. A dramatic loss of the high intensity CD45R positive cells occurred on the second day and returned to resting levels at each of the other time points. This is in contrast to CD4<sup>+</sup> T cells which showed variable expression of CD45R from day 0-12. The intensity of  $\alpha$ 4 integrin staining also increased on day 2.

CD8<sup>+</sup> T cells from sheep BT170, also showed increased intensity of expression for LFA-3 on day 4. No discernible difference was evident for CD2 staining. CD44 expression appeared to be down-regulated as was CD45R and L-selectin expression. Decreased levels of  $\alpha$ 4 integrin expression was observed on day 4. This is in contrast to the data for CD4<sup>+</sup> cells which showed increased expression of  $\alpha$ 4 integrin on day 4. The data from both these sheep would indicate that CD8<sup>+</sup> T cells during the secondary immune response show increased expression of the memory phenotype on day 2/4.

Levels of class II on CD8<sup>+</sup> T cells were also up-regulated. Increased DR and DQ expression was observed on day 2 and reached maximal levels by day 9 for sheep BT169 (Fig3.4b). Similarly elevated levels of class II expression were observed on day 4 for sheep BT170.

#### **5.2.4.3 $\gamma\delta$ T cells**

$\gamma\delta$  T cells do not express CD2 or the antigen recognized by the 73B mAb (anti-CD45R). Stimulation with ovalbumin appeared not to induce discernible variations in adhesion/activation markers on the circulating  $\gamma\delta$  T cells. This was evident from the data of both sheep examined. This correlates with the lack of difference observed between afferent and efferent lymph.

However increased expression of class II DR and DQ isotypes were observed in both animals examined. Similar to CD4<sup>+</sup> and CD8<sup>+</sup> T cells maximal expression was observed on day 9.

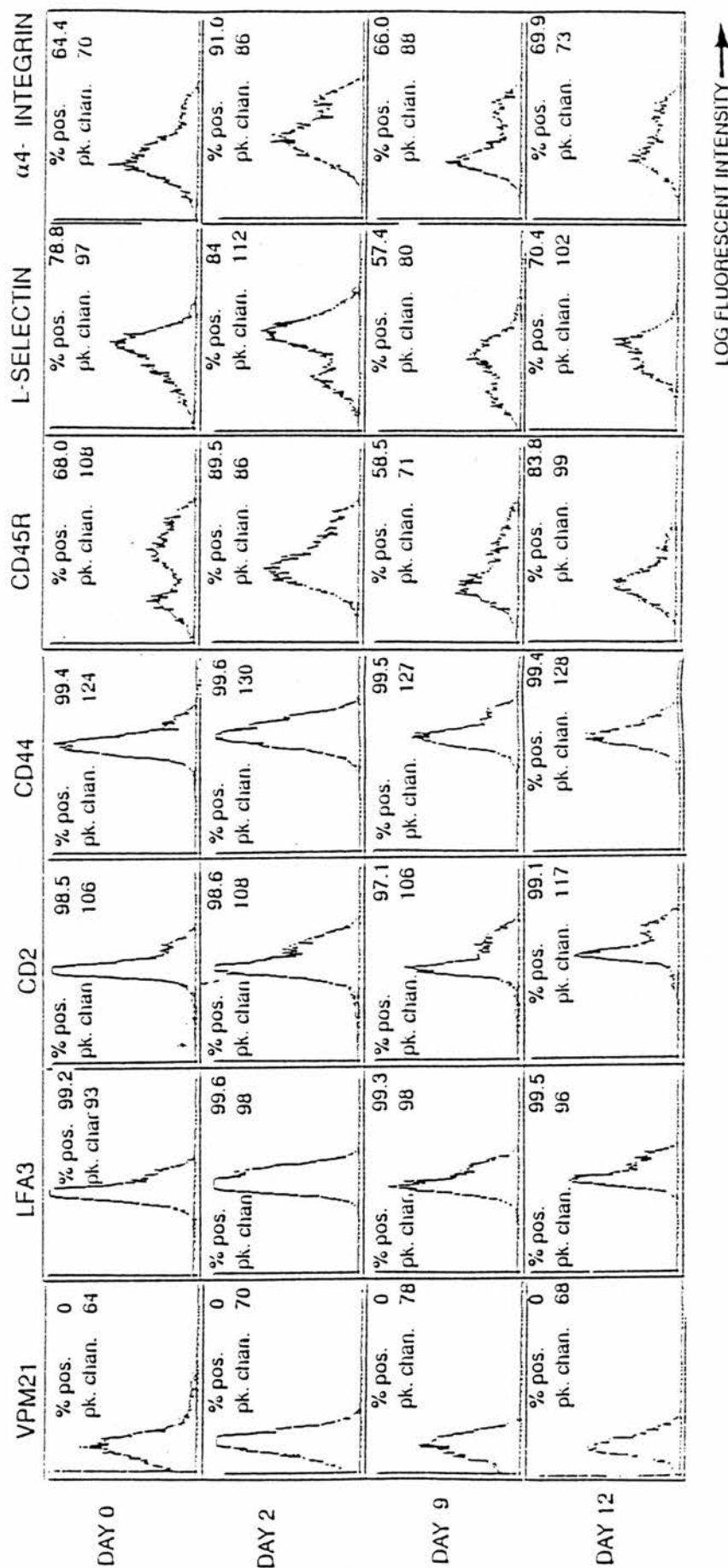
In summary, antigen challenge to the node resulted in increased expression of the memory phenotype on the circulating CD4 and CD8 positive T cells on day 2/4. This correlated with increased expression of class II molecules on these T cells. Class II expression remained up-regulated for a longer period. There were no observed changes in activation/adhesion molecule expression on  $\gamma\delta$  T cells, however increased expression of class II did occur.

Figure 5.4

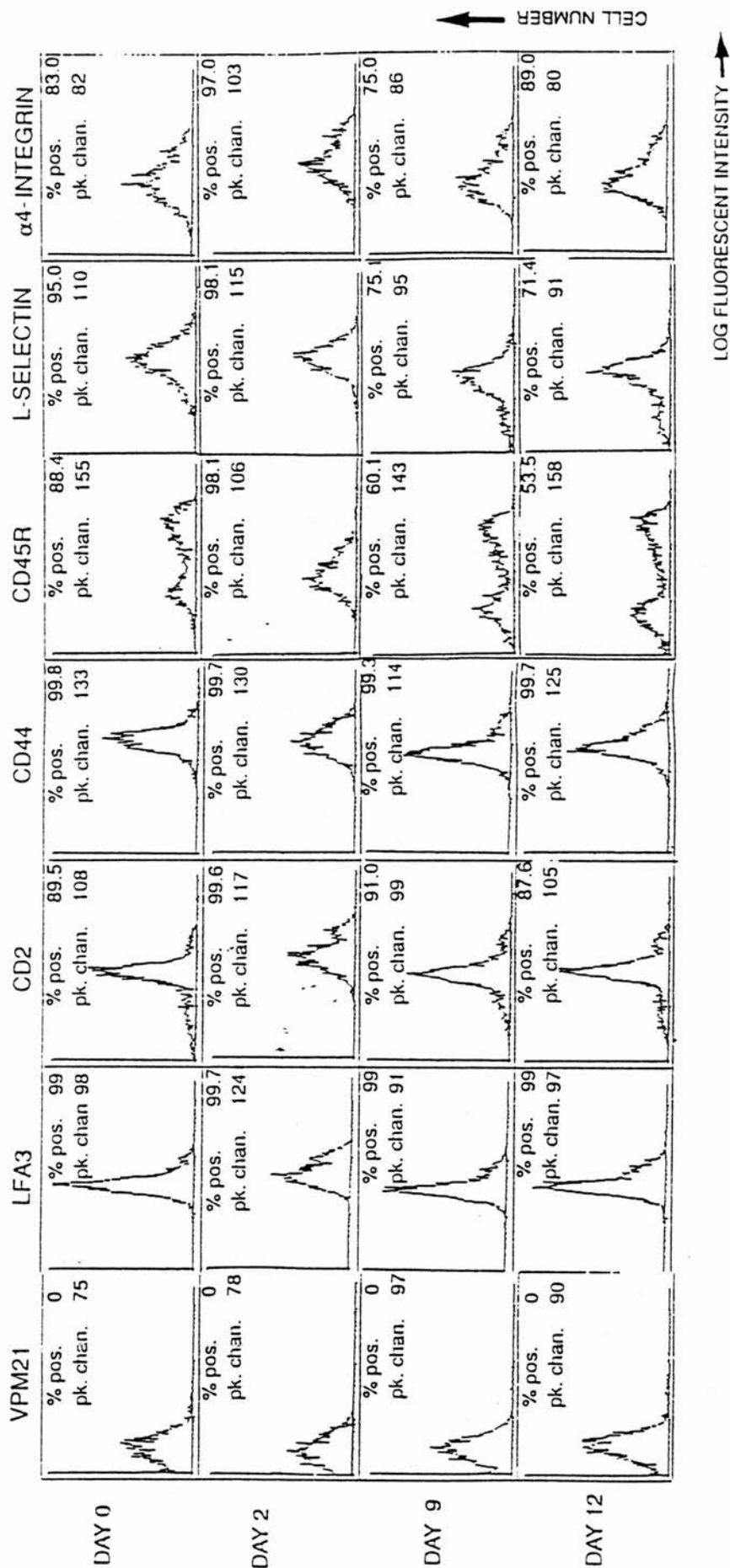
Expression of adhesion/activation markers on efferent lymph CD4<sup>+</sup> (A), CD8<sup>+</sup> (B) and T19<sup>+</sup> (C) T cells isolated during a secondary immune response to ovalbumin for sheep BT169. Cells were stained by two colour immunofluorescence and histograms represent single colour staining on gated CD4<sup>+</sup>, CD8<sup>+</sup> or T19<sup>+</sup> small lymphocytes. VPM21 staining on CD4<sup>+</sup>, CD8<sup>+</sup> and T19<sup>+</sup> T cells represents the negative control. The figures represent the percentage positive cells (% pos.) when compared with the negative control. Peak channel data (pk. chan.) is also shown.



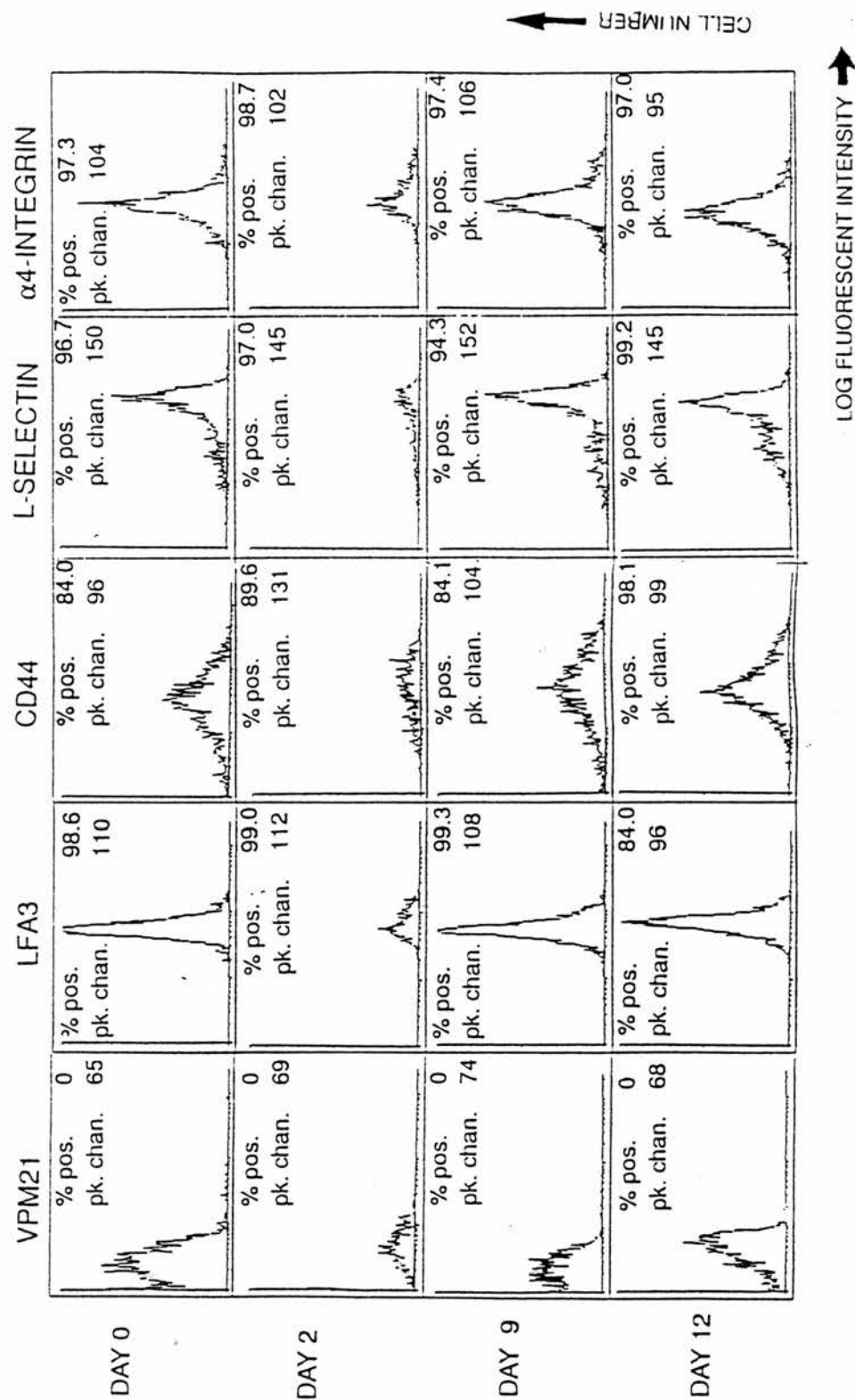
A



B



C

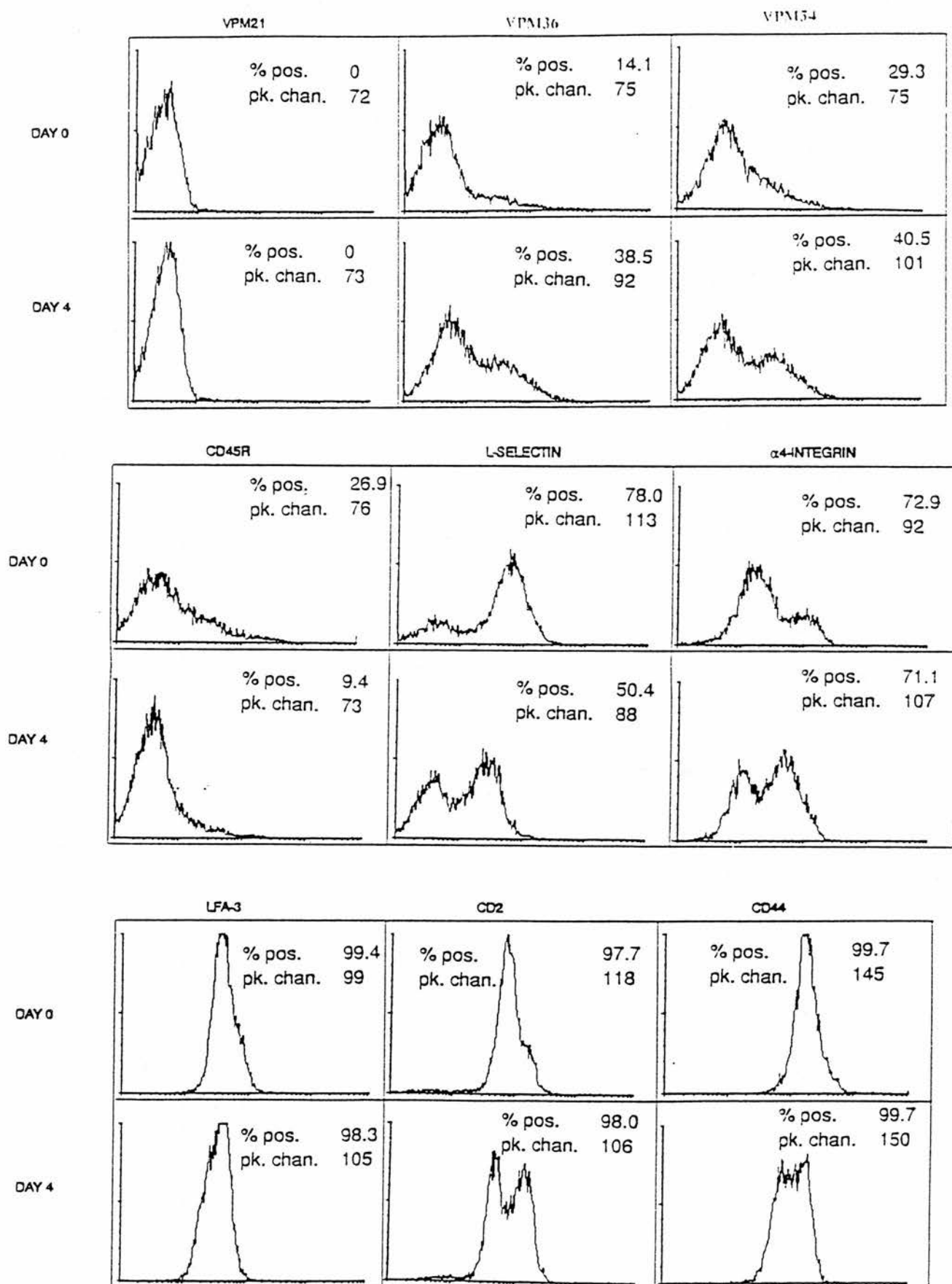


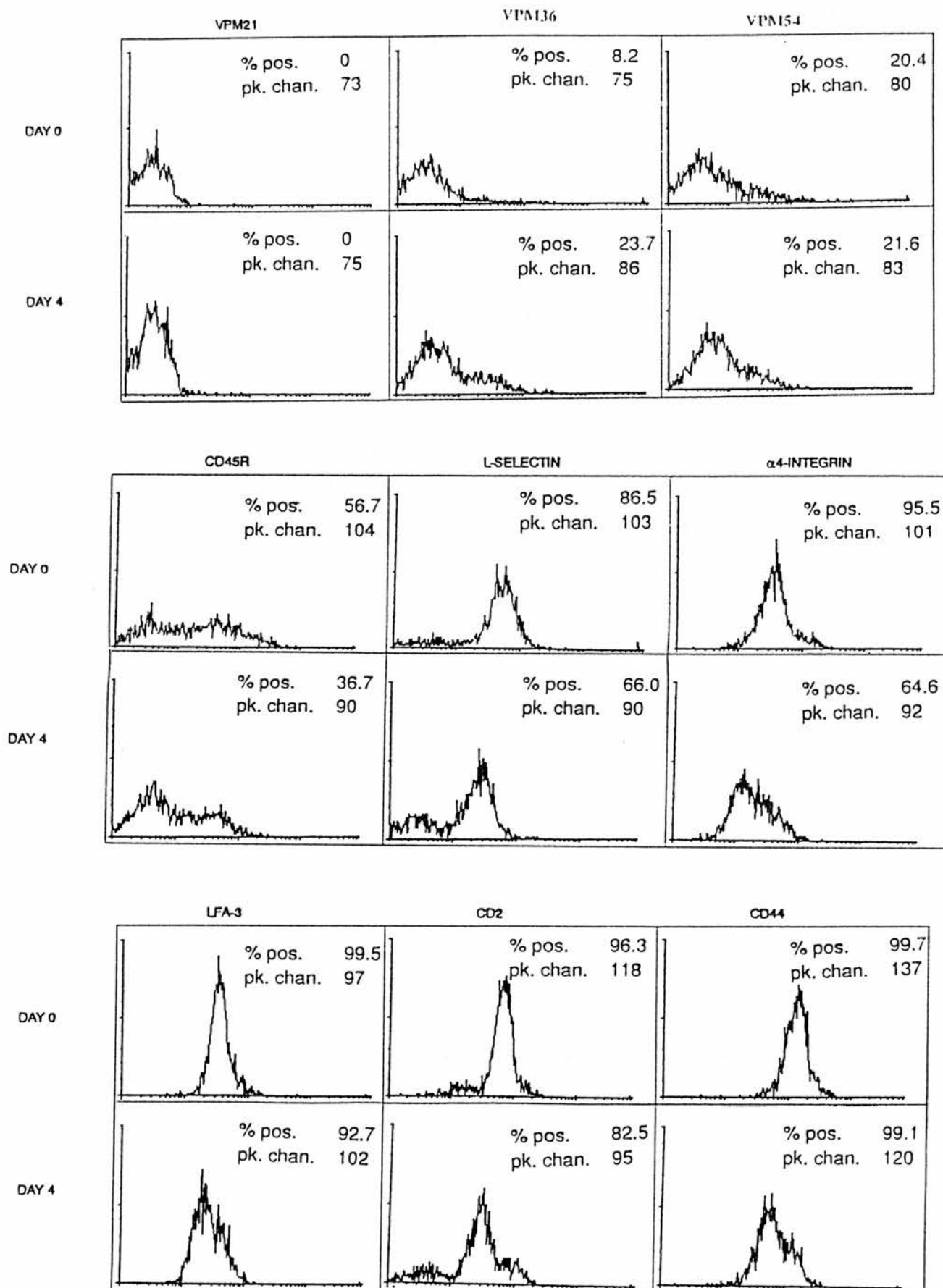


### Figure 5.5

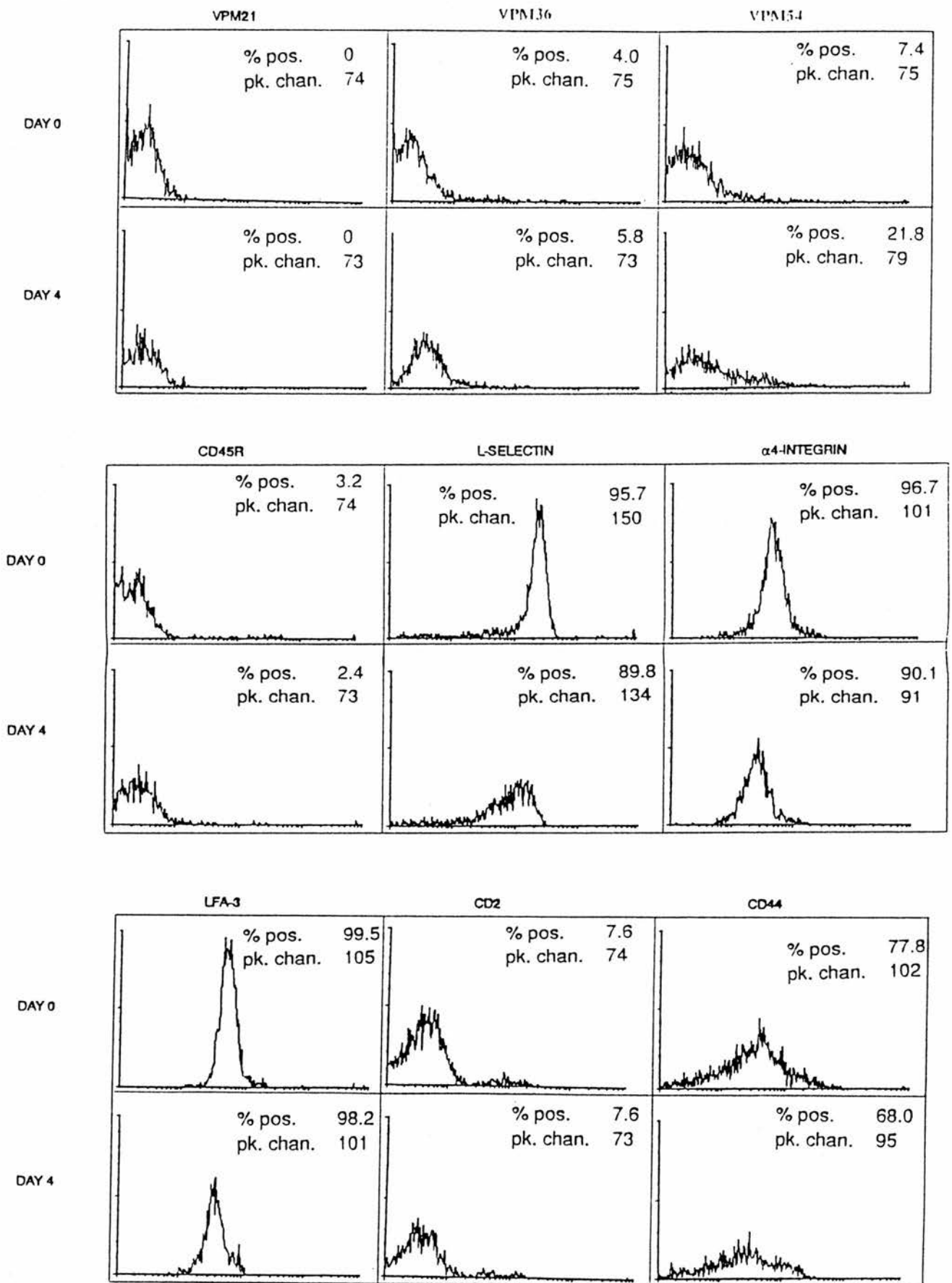
Expression of adhesion/activation markers and class II isotype expression on efferent lymph CD4<sup>+</sup> (A), CD8<sup>+</sup> (B) and T19<sup>+</sup> (C) T cells isolated during a secondary immune response to ovalbumin for sheep BT170. Cells were stained by two colour immunofluorescence and histograms represent single colour staining on gated CD4<sup>+</sup>, CD8<sup>+</sup> or T19<sup>+</sup> small lymphocytes. VPM21 staining on CD4<sup>+</sup>, CD8<sup>+</sup> and T19<sup>+</sup> T cells represents the negative control. The figures represent the percentage positive cells (% pos.) when compared with the negative control. Peak channel data (pk. chan.) is also shown.

**A**



**B**

C



### 5.3 Discussion

The association of T cell function with the expression of a unique array of cell surface determinants has led to a greater understanding of cell trafficking during immune responses. It has emerged that the migratory routes of memory/effector cells are different from those of naive precursor cells. T cells entering the lymph node via afferent lymph are phenotypically memory type, while those in the efferent lymph which derive mainly from the blood entering the LN across HEV are mostly naive phenotype (reviewed in Mackay, 1993). The studies described here on afferent and efferent lymph are consistent with these findings. Using mAbs to  $\alpha 4$  integrin, L-selectin and CD45R molecules, differences in their level of expression defined a reciprocal pair of T cell subsets. T cells of the afferent lymph were predominantly memory-type (L-selectin<sup>-</sup>, CD45R<sup>-</sup>  $\alpha 4$ -integrin<sup>hi</sup>) while in the efferent they were predominantly naive phenotype (L-selectin<sup>+</sup>, CD45R<sup>+</sup>,  $\alpha 4$ -integrin<sup>lo</sup>). Only marginal differences in levels of CD44 expression were observed between afferent and efferent lymphocytes and subdivision into high and low expressing populations as has been reported by Mackay *et al* (1990) was not carried out. Class II DR and DQ expression on CD4 and CD8 positive T cells coincides with the memory phenotype. The higher incidence of DR<sup>+</sup> DQ<sup>+</sup> T cells in afferent lymph coincides with the memory phenotype whereas efferent T cells are mostly class II negative, the DR<sup>+</sup> DQ<sup>-</sup> T cells may reflect the lower incidence of memory efferent T cells. This pattern was reflected on CD4<sup>+</sup> and CD8<sup>+</sup> T cell subsets.

The administration of antigen to a primed sheep enhances the traffic of lymphocytes through the regional lymph node. Increased numbers of CD4<sup>+</sup> and CD8<sup>+</sup> T cells appeared in the lymph. The composition of efferent lymph was characterized by an increased proportion of CD4<sup>+</sup> T cells during the first 3 days. This was followed by increased proportions of CD8<sup>+</sup> T cells on days 2 to 5. This is consistent with data published (Bujdoso *et al*, 1989). Profiles of activation/adhesion molecules were not obtained on a daily basis to correlate expression of adhesion/activation markers with exit of CD4 and CD8 positive T cells from the node.

The CD4 and CD8 positive T cell subsets during the immune response were

also characterized by increased expression of the memory phenotype markers (on day 2 for one sheep and day 4 for the second sheep). This is consistent with data published by Mackay *et al*, (1992). Variable expression of CD45R was observed over the time course for sheep BT169, while expression on sheep BT170 decreased. The significance of this event is not understood and may represent sheep to sheep variability. The increased expression of both DR and DQ class II molecules on day 2 correlates with the activation/memory phenotype. Elevated levels of activation/adhesion markers were evident at day 2/4, with the exception of L-selectin on CD4<sup>+</sup> T cells which displayed a memory phenotype until day 9. The increased expression of class II molecules was observed for up to 9 days following immune stimulation. Class II expression coincided with the increased expression of activation markers but the time courses of expression did not correlate. In comparison with activation/adhesion marker expression, levels of class II expression remained elevated for a longer period. Class II expression reached maximal levels on day 9, when activation/adhesion molecules had returned to resting levels. Because class II expression on T cell subsets was detectable for a longer period its presence may be indicative of a different functional role.

The panel of activation/adhesion molecules did not subdivide  $\gamma\delta$  T cells in afferent and efferent lymph into either memory or naive phenotype T cells respectively. Changes in the expression of activation/adhesion molecules was also not observed during the secondary immune response.  $\gamma\delta$  T cells did however show increased expression of both class II DR and DQ molecules. Because  $\gamma\delta$  T cells could not be identified as either naive or memory phenotype it was not possible to correlate activation status with class II expression.

No changes in numbers of circulating  $\gamma\delta$  T cells were observed during the immune response.  $\gamma\delta$  T cells appeared not to elicit an immune response to ovalbumin. In contrast, work published by Mackay *et al* (1992) reported changes in  $\gamma\delta$  T cell numbers following BCG challenge. The failure to detect changes may be a reflection on the antigen used. In mice, numbers of  $\gamma\delta$  T cells in the peritoneal cavity increase following *Listeria monocytogenes* (an intracellular bacterium) infection but not after *Nippostrongylus brasiliensis* (an extracellular parasite) infection (Ferrick *et al*, 1995).  $\gamma\delta$  T cells appear to be biased in their recognition of pathogens. A recent report, argues that  $\gamma\delta$  T cells have a system for specific T cell recognition that is alternate to the MHC presented peptide complex of  $\alpha\beta$  T cells (Tanaka *et al*, 1995). An antigen such as ovalbumin would not be detected by the  $\gamma\delta$

T cell whereas small non-peptidic antigens are recognized.

On activation both DR and DQ class II molecules were induced in each of the T cell subsets. DQ expression showing a marked induction on CD4<sup>+</sup> T cells. In sheep expression of DQ by T cells may be a result of recent activation (Dutia *et al*, 1993a) as has been implicated on human T cells (Oshima and Eckels, 1990a). However, correlations with other activation markers in this study did not provide further insights into the possibility that DR and DQ may fulfill different functional roles. In the next chapter the functional significance of class II isotypes will be examined with regard to cytokine secretion.



## CHAPTER 6 Correlation of MHC class II DR expression with cytokine expression.

### 6.1 Introduction

T cell activation in response to antigens involves the interaction of multiple receptor-coreceptor pairs which mediate cell adhesion and signal transduction. Having examined the correlation of the increased class II expression on activated T cells with adhesion/activation molecules, the relationship of class II DR expression with the outcome of signal transduction events is examined in this chapter. Signal transduction events that occur during antigen recognition by T cells eventually lead to the expression of cytokines which are mainly responsible for the mediation of cell function. It is the coordinate production of cytokines which is crucial for the regulation of an immune response. In the experiments described here, the relationship between class II DR expression and cytokine expression following activation was examined.

In recent years it has become evident that the immune response is largely dependent on the activation of particular subsets of CD4 cells that secrete defined patterns of cytokines. Initially, Mosmann *et al* (1986) subdivided mouse CD4<sup>+</sup> helper T cell clones on the basis of differences in their cytokine production. The subsets, termed Th1 and Th2 type T cells, showed distinct, and mutually exclusive, patterns of cytokine secretion. Th1 cells are defined as IL-2,  $\gamma$ -IFN and TNF $\beta$  secretors while Th2 cells produce mainly IL-4, IL-5, IL-6 and IL-10. Other cytokines such as TNF $\alpha$ , IL-3 and GM-CSF are produced by both Th subsets (reviewed in Mosmann and Coffman, 1989; Kelso *et al*, 1991; Janeway *et al*, 1988).

The existence of functional Th1 and Th2 subsets *in vivo* has been established using the mouse model of *Leishmania major* infection. Strains of mice that are highly susceptible to the parasite develop poor cell mediated immunity and produce Th2 type cytokines. In contrast, resistant mouse strains develop Th1 responses (Heinzel *et al*, 1989). A similar dichotomy has been found in humans using T cell clones expanded from patients suffering from leprosy. Th1 clones were derived from healed lesions of patients with tuberculoid leprosy and Th2 clones derived from patients with lepromatous leprosy (Salgame *et al*, 1991). The successful elimination of infectious agent depends on expansion of the appropriate CD4<sup>+</sup> T cell subset. In general Th1 cells are responsible for generating strong cellular

immunity against intracellular pathogens; Th2 cells promote the development of humoral responses that direct effector functions using specific antibodies against extracellular pathogens.

In the murine system Th1 and Th2 cells are repeatedly stimulated memory T cells that have differentiated from precursor T cells (Thp). Naive Thp cells produce IL-2 and progress into an intermediate Th0 stage in which the cytokines that characterize both Th1 and Th2 are produced. These cells then terminally differentiate into Th1/Th2 cells upon repeated antigen stimulation (Street *et al*,1990). Secretion of cytokines following activation is short-lived. Cytokine production occurs within a few hours. The cell then reverts to a resting state until further stimulation.

Once induced, the subsequent expansion of Th subsets is facilitated and regulated by subset specific cytokines. The dominant cytokines responsible for Th1 and Th2 differentiation are IL-12 and IL-4 respectively (reviewed in Paul and Seder,1994; Trinchieri,1993). IL-12 produced by macrophages in response to bacteria or parasites is an obligatory factor for Th1 generation and proliferation. IL-12 acts directly on Th1 cells and indirectly by inducing  $\gamma$ IFN production by T cells and NK cells.  $\gamma$ IFN has a positive feedback effect by positively inducing further IL-12 production. On the other hand, IL-4 generates a Th2 type response by inhibiting IL-2 receptor expression and  $\gamma$ IFN production. The Th2 cell products, IL10 and IL-4, have a negative feedback effect on IL-12 production (D'Andrea *et al*,1993).

Macrophages, NK cells and mast cells have been implicated in the directed development of the Th phenotype (Kullberg *et al*,1992). IL-12 is induced in macrophages infected with intracellular organisms, while IL-4 directed Th2 development may be through IL-4 production by CD4<sup>+</sup> T cells, mast cells and basophils.  $\gamma\delta$  T cells may also contribute to the cytokine milieu that influences the differentiation of antigen specific CD4<sup>+</sup> T cells into Th1 or Th2.  $\gamma\delta$  T cells have been shown to produce cytokines which are associated with the appropriate Th type response earlier than CD4 cells during infection (Ferrick *et al*,1995).

The immune response is directed by cytokines. However locally active hormones (Daynes *et al*,1991), antigen dose and antigen specific recognition (Murray *et al*,1994) can also control the functional outcome of immunity. Peptide

density on APCs has been predicted from MHC haplotype and cell mediated immunity was found associated with the MHC haplotype that displays high ligand density presentation *in vitro* (Murray *et al*,1994).

In sheep, as in humans, activated T cells expressing MHC class II are associated with cell proliferation and may have a regulatory role in the immune response. HLA-DR expressed by activated human CD4 T cell clones has been shown to play a role in signal transduction (Odum *et al*,1991). Antibody crosslinking of DR resulted in an increase in tyrosine phosphorylation and T cell activation. To understand the potential functional consequences of T cells expressing MHC class II, the cytokine profiles of unfractionated T cells and class II DR negative T cells stimulated *in vitro* was examined. Most of the initial knowledge on the regulation of cytokine production and the effect of these factors *in vitro* comes from studies performed with cell lines and T cell clones. Understanding the regulation and function of cytokine production by normal immunocompetent cells in *in vitro* situations mimicking immune responses, will give important information which may be more relevant to *in vivo* physiological function.

PCR (Saiki *et al*,1988; Kawasaki *et al*,1988) is currently the most sensitive technique for the detection of specific nucleic acid molecules present in low copy number. Quantitation of RNA can be achieved by coupling reverse transcription (Krug and Berger,1987) with PCR reactions (RT-PCR). Reverse transcription is employed to make first strand cDNA which is then used as a template for further amplification in PCR. Enhanced specificity and sensitivity can be achieved by hybridizing oligonucleotide DNA probes internal to the primers of the target sequence. The RT-PCR technique has been successfully applied to the precise quantitation of mRNA (Wang and Mark,1989) and the combination of RT-PCR and Northern blotting has allowed the comparison of relative amounts of specific mRNAs present in syngeneic and allogeneic cardiac transplants (Dallman *et al*,1991). Many of the ovine cytokines have now been cloned and so it was possible to use RT-PCR to provide a semi-quantitative analysis of cytokine mRNA levels.

Using RT-PCR, the kinetic analysis of  $\gamma$ IFN (Th1) production in *in vitro* concanavalin A activated efferent cells was established along with IL-6 and IL-10 (Th2) production by an LPS stimulated monocyte enriched cell population. Using the MACS, T cells were depleted of the MHC class II DR phenotype and the cytokine

profiles examined in the fractionated and unfractionated T cells following *in vitro* specific antigen and mitogen activation.

## **6.2 Results**

### **6.2.1 Kinetic analysis of cytokine production.**

The aim of these experiments was to establish the kinetics of  $\gamma$ IFN, IL-6 and IL-10 production from activated cell populations.  $\gamma$ IFN production was assessed in Con A activated efferent lymphocytes while IL-10 and IL-6 production was analysed from an LPS activated macrophage enriched population. Cytokines were assessed by semi-quantitative RT-PCR. An ovine ATPase alpha subunit PCR assay was used specifically for these studies (Woodall *et al*,1994). ATPase is a gene with a housekeeping function and therefore would be expected to be expressed in all cell types throughout development. Levels of cytokine expression were normalized with respect to ATPase expression. This approach gave a semi-quantitative measure of cytokine gene transcription.

#### **6.2.1.1 Cytokine profiles of Concanavalin A stimulated efferent cells.**

Efferent lymphocytes were cultured at  $2 \times 10^6$ /ml in the presence of 5 $\mu$ g/ml Con A. 10ml cultures containing  $2 \times 10^7$  cells were harvested prior to the addition of concanavalin A and 2, 4 and 8 hours following stimulation. The total RNA recovered was intact as determined by agarose gel electrophoresis (Fig 6.1a) with clear bands apparent for the 18S and 28S ribosomal RNA. mRNA is present as a smear between both these bands. cDNA was synthesized and PCR performed for  $\gamma$ IFN, IL-4, IL-10 and ATPase. Aliquots of the PCR reaction were removed after increasing numbers of cycles and analysed by slot-blot southern hybridisation (Fig 6.2).

The level of expression of ATPase is consistent in the 0, 2 and 8 hour time points with the gene detectable at 25 cycles in each sample. Expression of ATPase does not appear until 30 cycles in the 4 hour time point. Taking the ATPase levels as a measure of the amounts of mRNA per sample there is an increase in  $\gamma$ IFN expression after 2 hours of Con A stimulation, with maximal expression after 4 hours. IL-6 expression also appears after 2 hours, the lack of expression at 4 hours may be coincident with the lower levels of mRNA in this sample.

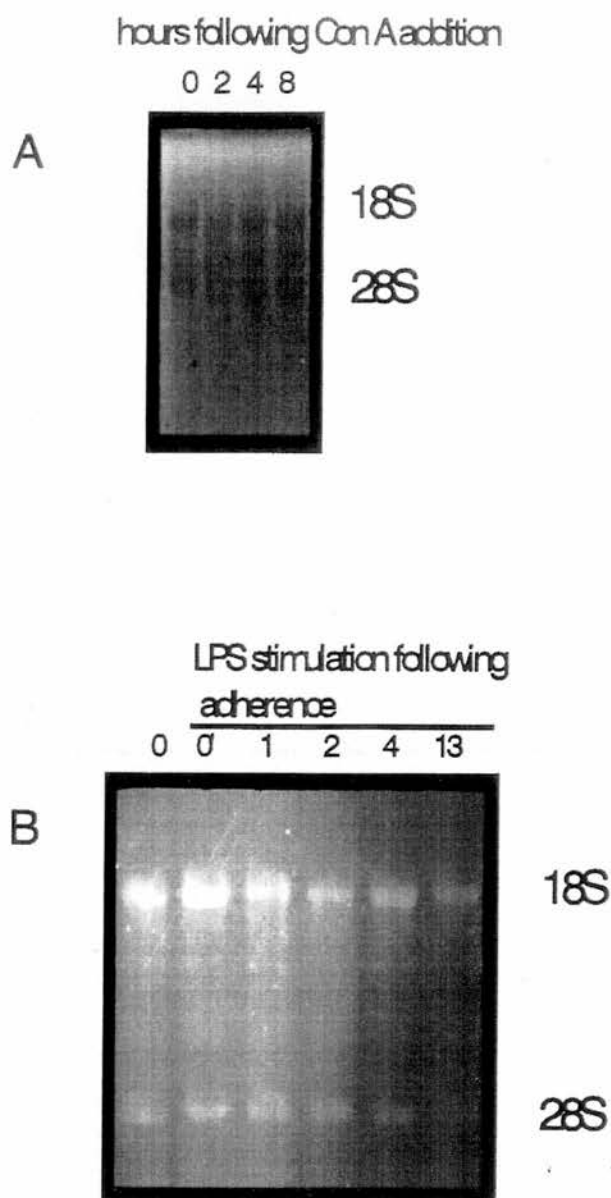


Figure 6.1

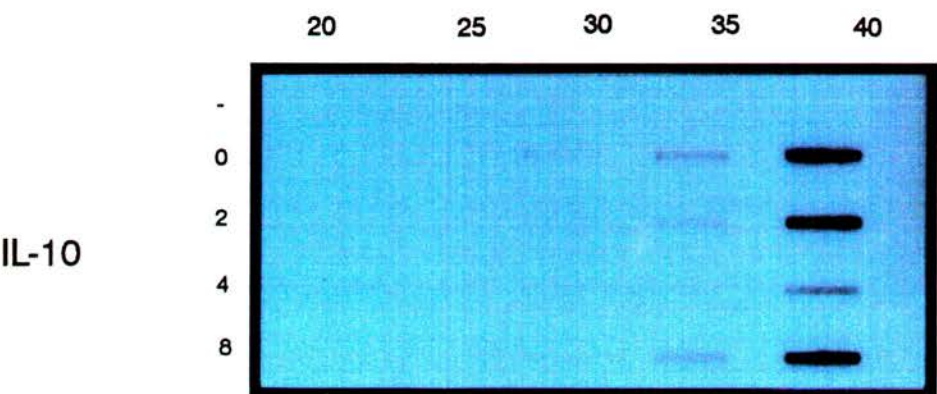
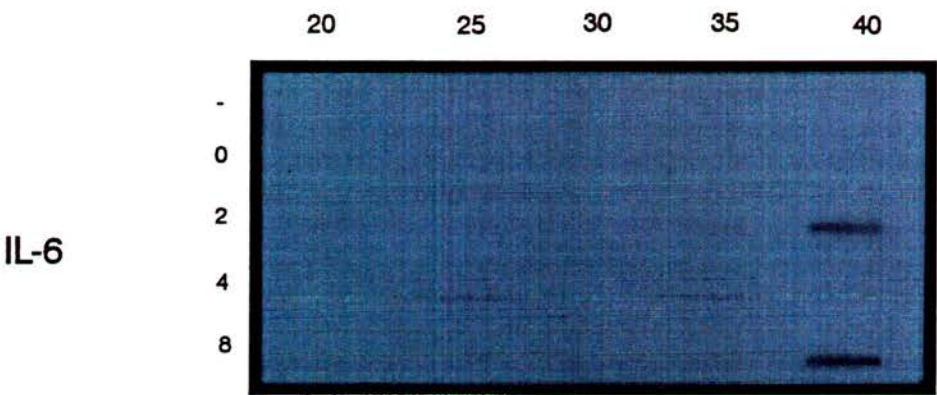
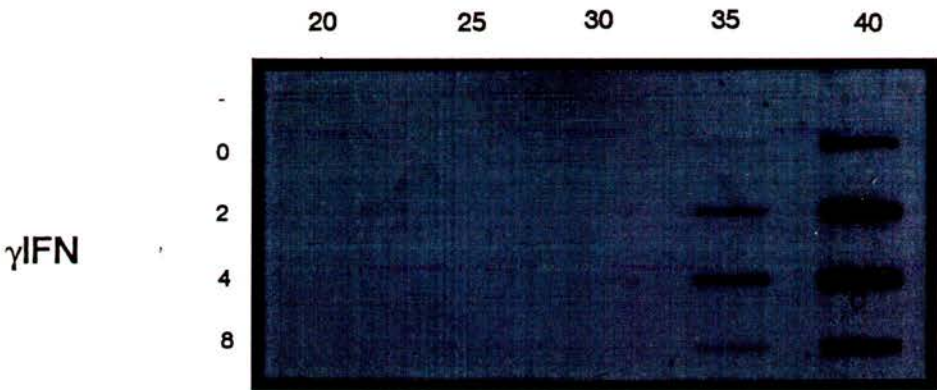
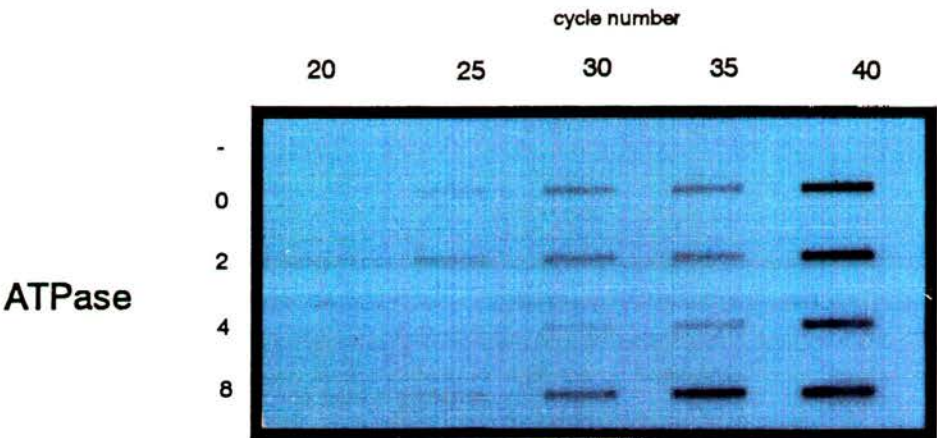
A. Analysis of RNA from concanavalin A stimulated efferent lymphocytes at 0, 2, 4 and 8 hour time points. 20 $\mu$ g of total cellular RNA per sample with the exception of the 2 hour sample which had only 10 $\mu$ g were fractionated on a 1% agarose/formaldehyde gel and visualised under UV.

B. Analysis of RNA from an alveolar macrophage enriched cell population before adherence (0), after adherence (0'), and 1, 2, 4 and 13 hours following LPS stimulation. 10 $\mu$ g of total cellular RNA per sample was fractionated on an agarose/formaldehyde gel and visualised under UV by ethidium bromide staining.

Figure 6.2

ATPase,  $\gamma$ IFN, IL-6 and IL-10 gene expression in concanavalin A activated efferent lymphocytes analysed by semi-quantitative RT-PCR. RNA was isolated, cDNA synthesised and 10  $\mu$ l PCR reaction mix was removed every 5 cycles from 20-40 cycles. PCR product was denatured and blotted directly onto nylon membrane. Blots were probed with  $^{32}$ P labelled internal oligonucleotide. Product was examined at 0, 2, 4 and 8 hours following concanavalin A activation. -, no cDNA control.







	$\gamma$ IFN		IL-6		IL-10	
	35 cycles	40 cycles	35 cycles	40 cycles	35 cycles	40 cycles
0hrs	0	0.92	0	0	1.52	3.6
2hrs	1.07	2.65	0	0.42	0.7	2.59
4hrs	1.88	4.09	0	0	0	0.72
8hrs	0.08	1.37	0	0.31	0.49	1.15

Table 6.1

Densitometric assessment of slot-blots shown in Figure 6.2. Values are presented as multiplication factors of the ATPase levels at each cycle. The data is of a single experiment.

There was little difference in the levels of IL-10 mRNA expression between the 0, 2 and 8 hour time points, again the 4 hour time point had a lower level. The data from the densitometer scan is normalized to ATPase expression per sample in Table 6.1. Values are given as ATPase multiplication factors. This data parallels the observations described.

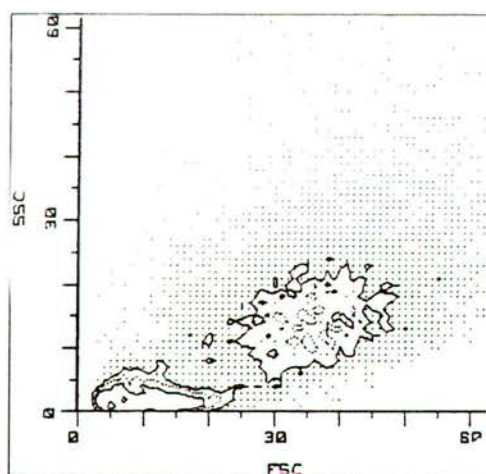
#### **6.2.1.2 Cytokine profiles of alveolar macrophage enriched cells stimulated with LPS**

Cells were recovered from ovine lungs as described in Chapter 2 (2.2.3.3). The recovered cells were set up in culture and macrophages allowed to adhere for 2 hours. The non-adherent cells were removed by washing and LPS at 10 $\mu$ g/ml added to the remaining macrophage enriched cells. mRNA was harvested from cells before adherence, after adherence and 1, 2, 4 and 13 hours after LPS stimulation. Fig 6.3 shows a flow cytometric profile and Leishmann stain of the population of cells prior to adherence. The flow cytometric profile indicates that this was not a homogenous macrophage population but contained lymphocytes as evident by the smaller size and granularity. The Leishmann stain shows a majority of macrophages, macrophages are large with many cytoplasmic granules, whereas lymphocytes have dense nuclei and sparse cytoplasm. The cells following adherence were not analysed.

There are particular problems in isolating mRNA from macrophages because of the abundance in lytic granulosomes. Cytokines are known to have AU rich sequences in the 3' untranslated region, conferring a short half-life on the RNA (Shaw and Kamen,1986). Therefore, with the disruption of the cellular microenvironment the RNA must be rapidly recovered. The integrity of the RNA was assessed under denaturing conditions of agarose gel electrophoresis with good RNA recovered for each sample (Fig 6.1b).

Figure 6.4 shows the slot-blot southern hybridisation after RT-PCR for IL-10, IL-6,  $\gamma$ IFN and ATPase. ATPase expression was detectable at 25 cycles in each sample indicating similar levels of mRNA.  $\gamma$ IFN levels were measured as a check on the purity of the population, because macrophages are not known to express  $\gamma$ IFN (Lau,1994). The levels of  $\gamma$ IFN were high in the initial samples prior to adherence when one can expect a large proportion of lymphocytes. Washing removed a proportion of lymphocytes and this was marked by reduced levels of  $\gamma$ IFN. Clearly a more rigorous procedure of washing is required to remove all lymphocytes and  $\gamma$ IFN production. Following adherence  $\gamma$ IFN was variably expressed.

A



B

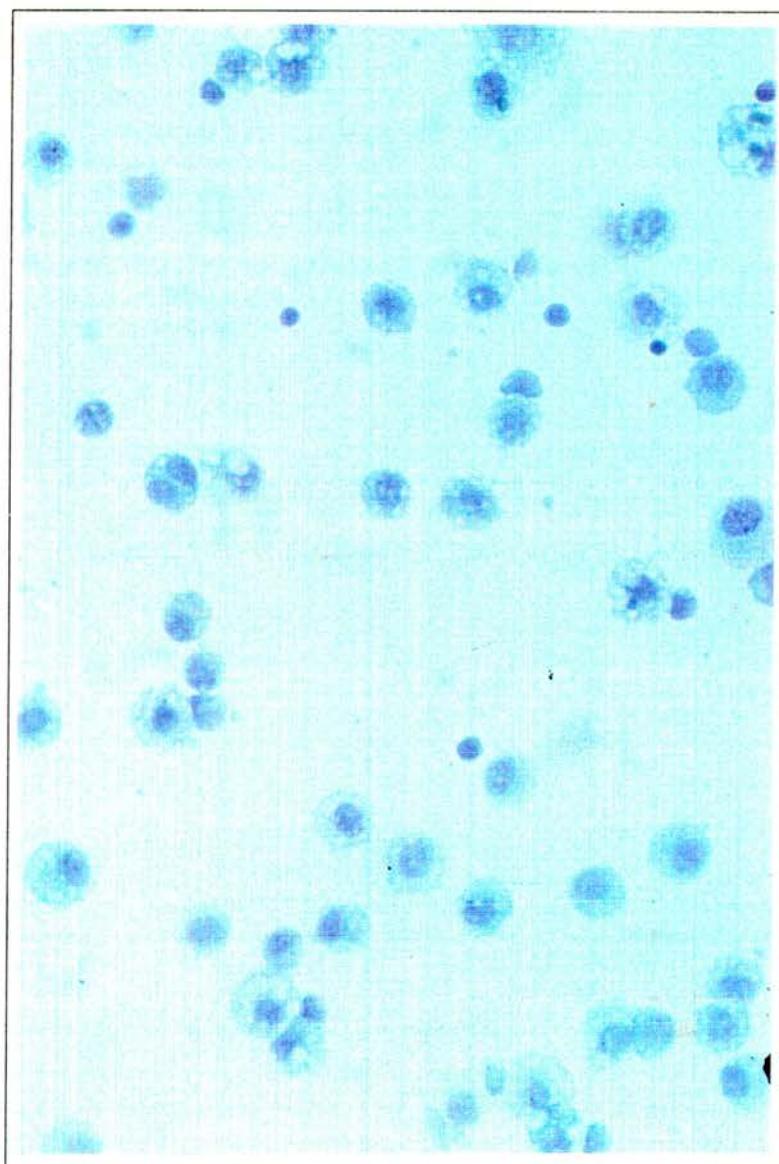


Figure 6.3

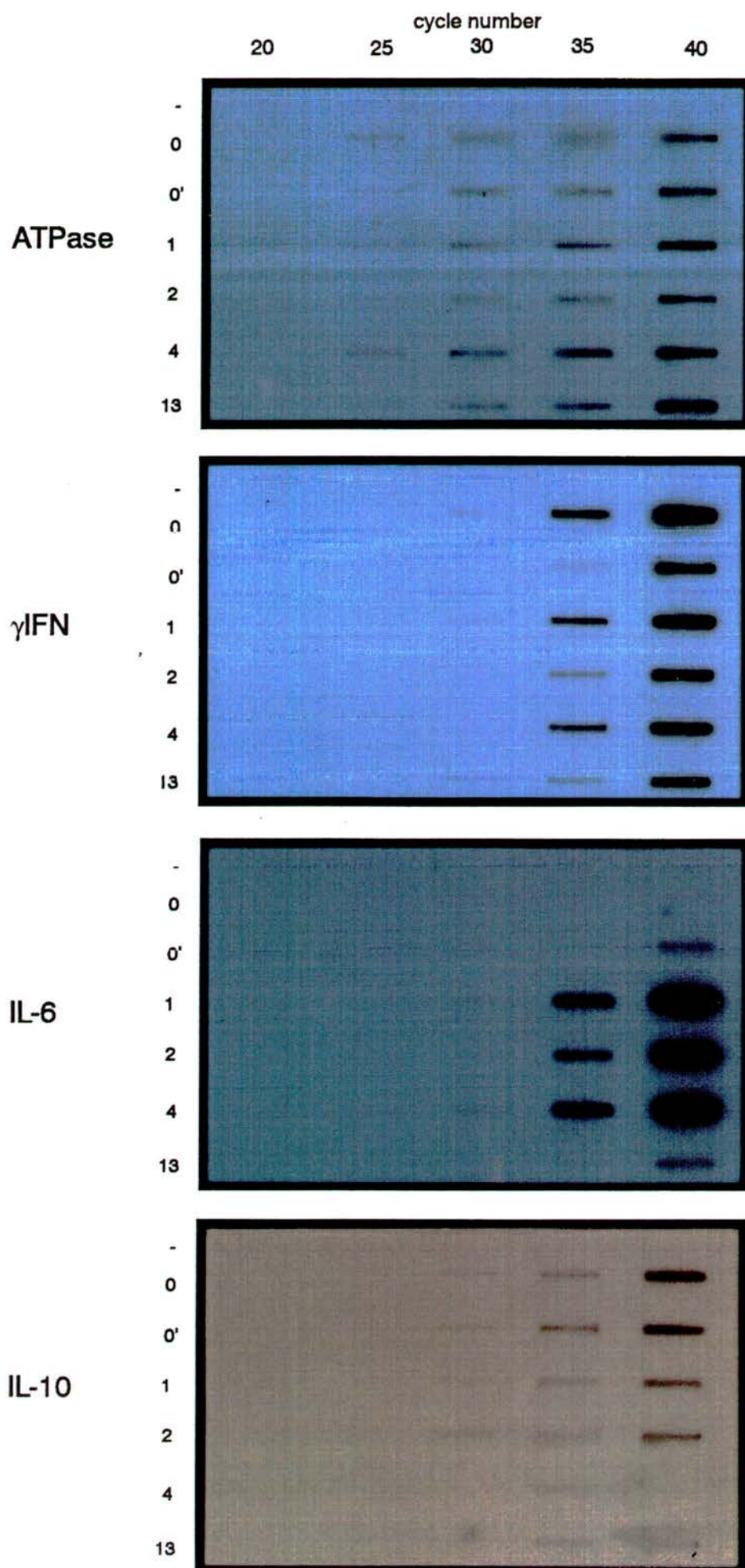
(A) Forward scatter and side angle scatter profile of the alveolar lavage cells prior to adherence to plastic.

(B) Leishmann stain of the same cell population.

Figure 6.4

ATPase,  $\gamma$ IFN, IL-6 and IL10 gene expression in LPS activated macrophage enriched alveolar cell population. Cells were harvested for mRNA and RT-PCR performed. Analysis was made at various time points following activation with LPS. -, represents the cDNA negative control.





	$\gamma$ IFN		IL-6		IL-10	
	35 cycles	40 cycles	35 cycles	40 cycles	35 cycles	40 cycles
0	3.32	7.03	0	0.14	0.23	1.12
0'	0.88	3.45	0	0.68	0.72	0.79
1	3.17	5.52	4.09	7.22	0.2	0.41
2	1.57	4.88	2.89	6.53	0.42	0.49
4	1.99	3.41	2.53	4.48	0.11	0.04
13	0.76	2.32	0	0.47	ND	ND

Table 6.2

Densitometric assessment of the slot-blots shown in Figure 6.4.

The levels of IL-10 show little variation over the time course. IL-6 is produced by both macrophages and lymphocytes and LPS has a stimulatory effect on both cell populations. At 0 time point there was very low expression of IL-6 mRNA. Adherence induces activation of macrophages which was marked by increased IL-6 mRNA expression. On LPS stimulation a clear enhancement in IL-6 mRNA levels could be detected as early as 1 hour. IL-6 mRNA expression was maximal after 1 hour and by 13 hours was beginning to decline. The data are normalized to ATPase expression in Table 6.2. As the level of ATPase expression was consistent in each sample the data can be used comparatively. Overall, IL-6 was dramatically induced following LPS activation, IL-10 levels showed little variation while  $\gamma$ IFN was variably expressed.

### **6.2.2 Activation of unfractionated T cells and DR<sup>-</sup> T cells.**

To examine the role of MHC class II isotype expression on T cells the cytokine profile of unfractionated T cells was compared with the profile from DR<sup>-</sup> T cells stimulated *in vitro*. Both antigenic and mitogenic stimulation was employed.

#### **6.2.2.1 Antigen stimulation of unfractionated T cells and DR<sup>-</sup> T cells.**

Efferent lymphocytes from an ovalbumin primed sheep were stimulated *in vitro* with ovalbumin using autologous irradiated PBMC as antigen presenting cells. Efferent lymphocytes were MACS purified into a total T cell population and a DR<sup>-</sup> T cell population (details of these experiments are given in 6.2.2.2). The purified T cells were established in culture at  $2 \times 10^6$ /ml with irradiated PBMC at a 1:1 ratio and ovalbumin at 500 $\mu$ g/ml. RNA was harvested from  $4 \times 10^7$  cells at various time points after the addition of antigen. As a control irradiated PBMC were set up in culture at  $4 \times 10^7$  cells/10mls and harvested for RNA prior to the addition of ovalbumin and 24 hours following addition of ovalbumin. RNA was purified, cDNA synthesized and ATPase primer pair added to the PCR reaction for product detection. The result is shown in Fig 6.5. Irradiation did not disrupt the levels of mRNA present in the APCs and ATPase was detectable at both time points in the irradiated cells. Following these results it was decided to stimulate T cells non-specifically with Con A to examine cytokine profiles.



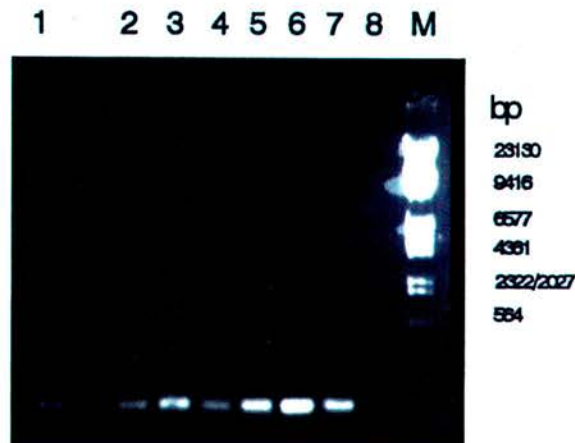


Figure 6.5

ATPase PCR product fractionated by electrophoresis through an ethidium bromide agarose gel. Samples are as follows

Lane1 and 2, amplified product from  $10^7$  efferent cells MACS fractionated into DR<sup>-</sup> T cells with autologous irradiated PBMC at a 1:1 ratio. Cells were stimulated with ovalbumin and analysed at either 0 or 24 hours.

Lane 3 and 4, amplified product from  $2 \times 10^7$  irradiated PBMC at 0 and 24 hours following the addition of ovalbumin.

Lane 5, 6 and 7, amplified product from  $10^7$  unfractionated efferent cells with an equal number of autologous irradiated PBMC. Cells were stimulated with ovalbumin and analysed at 0, 4 and 24 hours.

Lane 8 represents the no cDNA control.

#### 6.2.2.2 Mitogen stimulation of unfractionated T cells and DR<sup>-</sup> T cells.

The initial plan was to isolate T cell subsets into DR and DQ expressing populations using the MACS. However, from the results described in Chapter 4, it was not possible to isolate T cell subsets negatively. This was due to the lack of a null cell specific mAb. Positive selection with the MACS was deemed unsuitable, as antibody binding may affect the resultant cytokine profile. Thus it was decided to prepare a total T cell population from efferent lymph by negatively removing B cells, macrophages, monocytes and granulocytes with the MACS. By further removing DR<sup>+</sup> T cells from the isolated T cell population, it was possible to compare the cytokine profile of the DR<sup>-</sup> T cell population with the total T cell population. Isolation of a DQ<sup>-</sup> T cell population was not examined as DQ is expressed on only a small percentage of unstimulated T cells.

Lymphocytes were purified from defibrinated blood by hypotonic lysis of the red blood cells followed by centrifugation over lymphoprep. These cells were used for MACS purification of a T cell population and a DR<sup>-</sup> T cell population. Biotinylated antibodies to granulocytes/monocytes [VPM65 (anti-CD14) & VPM64 (anti-CD16)] and B cells (VPM30) were used to remove these cells, leaving a pure T cell population. To purify the DR<sup>-</sup> T cells, biotinylated VPM54 (anti-DR $\alpha$ ) was used to remove DR<sup>+</sup> cells. Flow cytometric profiles of the selected cells are shown in Fig 6.6. The purity of the cells isolated by MACS was >95% as determined by lack of phycoerythrin expression. The purified cell populations were stained with neither a pan T cell marker or a DR specific antibody to establish unequivocally their purity. The selected cell populations were cultured at  $2 \times 10^6$  cells/ml with Con A at 5 $\mu$ g/ml.  $2 \times 10^7$  cells were harvested for RNA extraction at 0, 2, 4 and 16 hours using the total T cell population. As fewer cells were recovered for the DR<sup>-</sup> T cell population,  $2 \times 10^7$  cells were harvested at two time points (0 and 4 hours) for RNA analysis. Less than 0.3mg RNA was recovered, this was insufficient for agarose gel electrophoresis. cDNA was synthesized and the genes detected by RT-PCR. Samples were standardized according to the percent incorporation of <sup>32</sup>P-dCTP. The results of the slot-blot southern hybridization are shown in Fig 6.7 and the data from densitometric scans is presented in Table 6.3. ATPase was detectable at 25 cycles in all samples with higher levels in the 0 and 2 hour time points of the total T cell population. Values shown in Table 6.3 are normalized to ATPase levels.

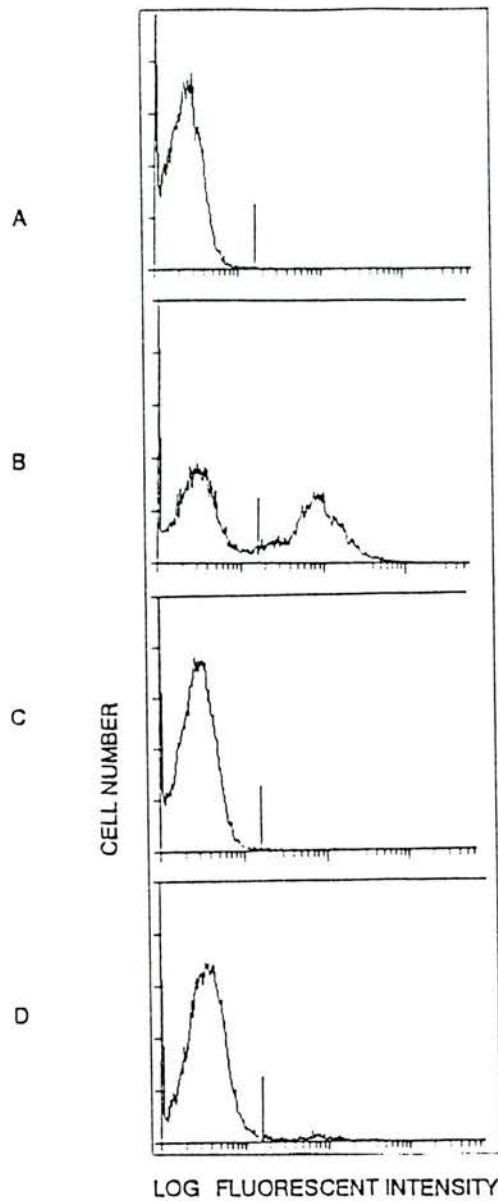


Figure 6.6

Flow cytometry histograms of PBMC purified by MACS. T cells were purified by negative selection using mAbs to B cells (VPM30) and granulocytes/monocytes (VPM65 & VPM64). The T cells were further purified by removal of the DR<sup>+</sup> cells using VPM54. Positive staining represents the cells stained with both supramagnetic beads and phycoerythrin. Cells were not gated on their scatter profiles. The figures indicate the percentage of positively stained cells.

(A) unstained PBMC

(B) cell staining prior to MACS separation,

(C) staining pattern of the negatively selected T cell population,

(D) staining pattern of the negatively selected DR<sup>-</sup> T cell population.



Densitometric assessment may not be completely accurate as bands on a blot that contain large but different amounts of PCR product will appear of the same intensity even after short exposure on X-ray film. Hence both values at 35 and 40 cycles are shown.

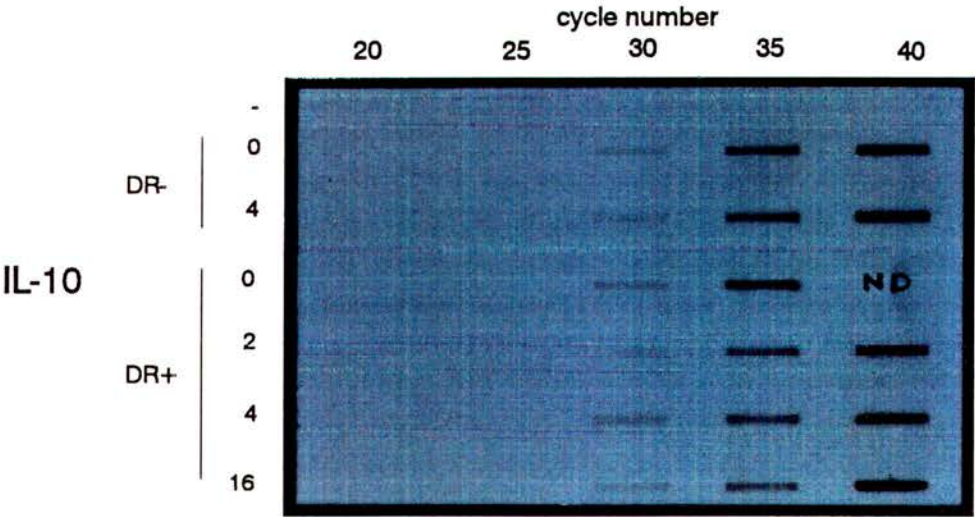
$\gamma$ IFN mRNA levels increased following Con A activation in the total T cell population. Over the time course examined there was maximal expression at 16 hours.  $\gamma$ IFN levels showed little increase in expression during the first 4 hours. Likewise, the DR<sup>-</sup> T cell population showed no marked increase/decrease in  $\gamma$ IFN levels after stimulation. Maximal expression was detected at 16 hours, which is in contrast to the earlier result of efferent cells which expressed  $\gamma$ IFN maximally after 4 hours of Con A activation.

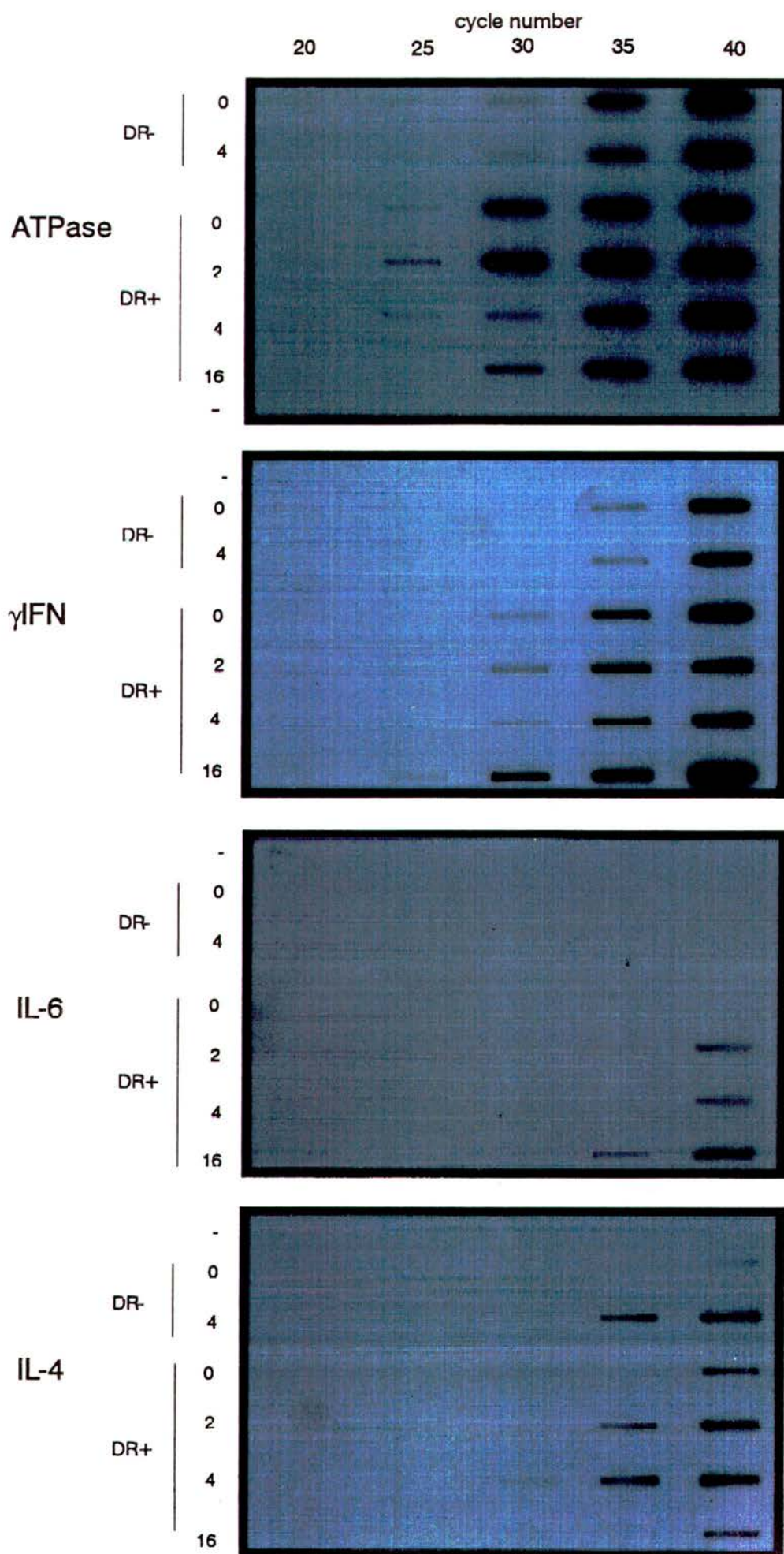
IL-6 mRNA was detectable 2-16 hours after Con A stimulation in the total T cell population. The densitometer values at 35 cycles indicate that levels of IL-6 mRNA expression were much lower than that of the other cytokines examined. In contrast to the total T cell population, there was no induction of IL-6 production in the DR<sup>-</sup> T cell population after Con A activation. Con A stimulation induced IL-6 production in the unfractionated T cell population but did not induce IL-6 production in the DR<sup>-</sup> T cell population.

As IL-6 gave such an interesting result it was decided to look at another Th2 type cytokine and IL-4 was chosen. IL-4 mRNA was detectable in all samples with increased expression in both populations over 4 hours. The densitometer values show similar levels of IL-4 after 4 hours in both populations. In the total T cell population the IL-4 mRNA levels had declined to resting level by 16 hours.

IL-10 mRNA levels showed no variation in levels of expression in either of the T cell populations. IL-10 expression was detected as early as 30 cycles in each sample and appeared to be expressed at similar levels throughout. The detectable expression of IL-10 at 30 cycles would indicate that IL-10 was more highly expressed than either IL-4 or IL-6.

Figure 6.7  
 $\gamma$ IFN, ATPase, IL-10, IL-6 and IL-4 gene expression on MACS purified T cells and DR- T cells (MACS purification was described in Figure 6.6). Cells were cultured and stimulated with concanavalin A. RNA was extracted from the unfractionated T cells (total DR+) at 0, 2, 4, and 16 hours following activation and at 0 and 4 hours following activation in the DR- T cell population. - represents the cDNA negative control





	$\gamma$ IFN		IL-6		IL-4		IL-10	
	35 cycles	40 cycles	35 cycles	40 cycles	35 cycles	40 cycles	35 cycles	40 cycles
DR- 0	0.2	1	0	0	0	0.01	0.52	0.27
DR- 4	0.25	0.71	0	0	0.26	0.23	0.5	0.35
DR+ 0	0.57	0.95	0	0	0	0.1	0.22	ND
DR+ 2	0.55	0.49	0	0.11	0.09	0.25	0.12	0.22
DR+ 4	0.49	0.55	0.05	0.17	0.21	0.4	0.23	0.24
DR+ 16	1.31	1.32	0.17	0.45	0	0.08	0.23	0.28

Table 6.3

Densitometric assessment of the slot-blot shown in Figure 6.7.



### 6.3 Discussion

In an attempt to understand the functional significance of class II positive T cells, the cytokine profile of a DR<sup>+</sup> T cell population was compared with unfractionated T cells (a proportion of which express the DR isotype). The interesting finding was that IL-6 is induced on activation only in the total T cell population and not in the DR<sup>+</sup> T cell population. Production of IL-6 mRNA correlated with DR expression by T cells. IL-6 is a cytokine with pleiotrophic activities. IL-6 is involved in the terminal differentiation of B cells into antibody producing plasma cells, the induction of acute phase proteins, the differentiation/activation of T cells and the induction of IL-2 and IL-2 receptor expression on T cells (reviewed in Akira *et al*, 1994; Lau, 1994). It has also been found that IL-6 can replace the requirement for APC derived costimulatory signals for IL-2 secretion and proliferation (Kasahara *et al*, 1990; Lorre *et al*, 1990).

It is this last point which may be the key to the significance of IL-6 secretion by the DR positive T lymphocytes. Despite the expression of large amounts of MHC class II following activation, these cells do not induce antigen specific T cell proliferation (Hopkins *et al*, 1993). The increased expression of DR may act to transduce signals for IL-6 production. The activation and production of IL-6 may allow the proliferation and differentiation of naive T cells (DR<sup>+</sup>) without the need for APC stimulation. IL-6 has been shown to induce IL-2 and IL-2 receptor expression. In studies conducted by Odum *et al* (1993), it was found that DR expressing T cells showed increased IL-2 receptor expression and IL-2 sensitivity after activation. The production of IL-6 by DR<sup>+</sup> T cells may induce IL-2 receptor expression on T cells, thus amplifying the specific immune response locally.

IL-6 is produced by many cell types (monocytes, macrophages, fibroblasts, T cells, B cells, granulocytes, mast cells, endothelial cells and osteoblasts) (Akira *et al*, 1994) and because the purity of the isolated T cell populations was not established care must be taken in drawing conclusions from this data. Non T cells present may have contributed to the production of IL-6.

The subdivision of T cells into Th1 and Th2 types was based on the cytokine profiles of T cell clones *in vitro*. The cell population used in this study was derived from sheep PBL and as such will represent an array of Th0, Th1 and Th2 type cells.

These *ex vivo* cells may display a diversity in their cytokine profiles such that the Th1/Th2 division represents extremes of the phenotype (Kelso,1995). This may explain why production of IL-4 mRNA by the DR<sup>+</sup> T cell population did not correlate with IL-6 production, both of which are Th2 type cytokines.

Con A activation of efferent cells induced the synthesis of  $\gamma$ IFN mRNA and by 4 hours expression was maximal. This is in agreement with work carried out by Henfrey (1993). In the purified T lymphocyte population isolated from peripheral blood, Con A did not induce maximal expression of  $\gamma$ IFN until much later (16 hours). This is perhaps a consequence of the absence of APC costimulatory signals in the pure T cell population. Concanavalin A stimulates T cell proliferation in the absence of MHC molecule recognition. It is presumed to interact directly with the CD3 complex. In the absence of accessory cells, T cell populations show a significant reduction in mitogen responsiveness. This is consistent with the need for accessory cell-derived second signals for optimal proliferative response (Mueller *et al*,1989). The costimulation required is delivered by the APC and involves the CD80 (B7) molecule on the APC interacting with the CD28 and CTLA-4 receptors on T lymphocytes (Linsley *et al*,1991a & b)

Con A can induce stimulation which may also induce cytokines not detectable with antigen stimulation. In the experiments described the T cells were activated nonspecifically with Con A. Attempts to study the cytokine profile following activation through the TCR proved problematic. It was found that irradiated APCs produced cytokine specific mRNAs. Thus T cell production of cytokine RNA following antigen stimulation by inactivated APCs could not be assessed. Perhaps higher levels of irradiation would destroy the RNA but then such levels may hinder the ability of the cells to present antigen. Actinomycin D inhibits transcription of RNA by binding tightly and specifically to double-helical DNA. This prevents it from being an effective template for RNA synthesis. Use of actinomycin D may have been a better choice at blocking mRNA synthesis without affecting antigen presentation. Due to time constraints, I was unable to set up the control experiments for the use of actinomycin D.

Macrophages produce IL-10 in response to LPS and this has an inhibitory effect on the LPS induction of other cytokines (Fiorentino *et al*,1991). However, the results described for the alveolar macrophage enriched cell population stimulated with LPS show low levels of IL-10 production. As this was not a pure

macrophage population, cytokines induced in the other cells present may have contributed to the reduced IL-10 levels.  $\gamma$ IFN and IL-10 have been found to regulate each others production and function in a negative manner. Monocyte depletion from *Staphylococcus aureus* activated PBMC reduced IL-10 production by 90% and resulted in increased  $\gamma$ IFN production (Chomarat *et al*,1993). This possibly explains the results of cytokine mRNA levels observed in the LPS activated alveolar monocyte enriched cells. The low level of IL-10 mRNA throughout the time-course may be attributed to the antagonistic effect of  $\gamma$ IFN which was highly expressed. The inhibitory effect of  $\gamma$ IFN production on IL-10 production by macrophages highlights the interplay of cytokines in regulating immune responses. The IL-10 mRNA levels expressed by the purified T cells in comparison were much higher, but again showed little variation over the time-course, this may also be attributed to  $\gamma$ IFN down-regulation.

Precise quantitation of nucleic acids by RT-PCR is problematic as the procedure involves two enzymatic steps with the second PCR step having an exponential nature. Variations in efficiency of the cDNA synthesis coupled with variations in amplification efficiency dramatically affect the yield of amplification product. The use of an internal control template was first described by Chelly *et al* (1990) to which target RNA levels can be normalized allowing the direct comparison of RNA in separate samples. Ovine ATPase was used as an internal control for sample-to-sample variations in both the RT and PCR reactions in these experiments. It is ubiquitously expressed and acts to maintain the sodium potassium ion balance across the plasma membrane playing a fundamental role in cellular metabolism. However it is not known if there is invariant expression following activation of cells. It would have been interesting to carry out Northern blots of the RNA samples and hybridize with labelled double-stranded ATPase probe to confirm the levels observed with RT-PCR. Such an approach would be more quantitative and would possibly answer the question of invariance in ATPase expression.

## CHAPTER 7 Concluding remarks

There are striking differences between species in the expression of MHC class II on T cells. In humans and rats, T cells express class II only after activation (Oshima and Eckels, 1990a; Broeren *et al*, 1995), while in the sheep a proportion of T cells are class II positive and increased expression occurs after activation (Hopkins *et al*, 1986 & 1993). Equine (Crepaldi *et al*, 1986), canine (Doveren *et al*, 1986) and feline (Rideout *et al*, 1992) T cells constitutively express class II. The functional significance of class II positive T cells is unclear. T cells can present peptide antigens and function as antigen presenting cells in MLR, but their ability to present soluble antigen is limited by their inability to take up antigen. In humans, class II positive T cells have been implicated in the induction of anergy (O'Hehir and Lamb, 1990; LaSalle *et al*, 1992) and in signal transduction (Odum *et al*, 1991 & 1993). In rats, expression of class II on T cells has been shown to be involved in T-T cell regulation (Broeren *et al*, 1994). The work described in this thesis was conducted to examine the functional significance of class II positive ovine T cells.

Monoclonal antibodies specific for sheep homologues of DQ and DR were characterized. The amino terminal sequence analysis of the  $\alpha$  chains recognized by VPM36 and VPM54 show a high degree of homology to the DQ and DR  $\alpha$  chains in humans. This allowed the use of these mAbs in the unequivocal identification of the ovine equivalents of the human DQ and DR locus products. Both mAbs were then used to correlate T cell expression of class II isotypes with T cell activation status and cytokine synthesis profiles.

Phenotypic differences between afferent, efferent and peripheral blood show that afferent lymph T cells are mainly of the memory phenotype, while efferent and peripheral blood contain a mixture of naive and memory T cell phenotypes (Mackay *et al*, 1990). There are no definitive markers of memory or naive T cells and the expression of activation and adhesion molecules is used to define different cell phenotypes. Because the relationship of class II expression has not been correlated with other markers of activation, studies were conducted to correlate these events on T cell subsets derived from the various immunological compartments. Variable expression of class II on T cell subsets between afferent and efferent lymph was found. Afferent T cell subsets (CD4<sup>+</sup>, CD8<sup>+</sup>) expressed increased levels of both DR and DQ when compared with levels of expression on efferent lymphocytes. Afferent T cells were also characterized as memory phenotype (CD45R<sup>-</sup>, L-selectin<sup>lo</sup> and

$\alpha 4$ -integrin<sup>hi</sup>). Thus the DR<sup>+</sup>DQ<sup>+</sup> phenotype of afferent CD4<sup>+</sup> and CD8<sup>+</sup> T cells correlated with a memory phenotype. The T cells derived from efferent lymph were predominantly DR<sup>-</sup>DQ<sup>-</sup> and this correlated with a naive phenotype (CD45R<sup>+</sup>, L-selectin<sup>hi</sup>,  $\alpha 4$ -integrin<sup>lo</sup>). The DR<sup>+</sup>DQ<sup>-</sup> T cells of efferent lymph may reflect a low incidence of memory efferent T cells. It appears that class II expression correlates with a memory phenotype on CD4<sup>+</sup> and CD8<sup>+</sup> T cell subsets.

Studies in humans and sheep report increased expression of class II molecules after activation (Evans *et al*,1978; Ko *et al*,1979; Hopkins *et al*,1986). To further establish the relationship of class II expression with activation status, class II expression and activation/adhesion marker expression was correlated after *in vivo* antigenic challenge.

Antigen challenge to the lymph node has been likened to the events that occur during inflammation (Mackay *et al*,1992). For example, during inflammation the phenotype of the endothelium is dramatically modified. These changes are due to the production of inflammatory cytokines IL-1, TNF and  $\gamma$ IFN which induce cell surface expression of adhesion molecules on the endothelia. These events promote the recruitment of memory lymphocytes to the inflammation site. After antigen challenge to the node, increased traffic of T cells expressing the memory phenotype are found to exit the node in the efferent lymph (Mackay *et al*,1990). These observations are only detectable in secondary immune responses. The primary immune response takes time to develop because the novel antigen must be screened by T cells before establishing a specific response. In contrast, the secondary response is more rapid due to the presence of specific memory cells. Production of inflammatory cytokines assist in the tissue selective homing of antigen specific memory T cells (Mackay *et al*,1992) which have a minimal requirement for costimulation (Bradley *et al*,1993).

Consistent with these observations the *in vivo* secondary immune response described in this study was characterized by increased proportions of both CD4<sup>+</sup> and CD8<sup>+</sup> T cells leaving the node. As increased proportions of CD4<sup>+</sup> and CD8<sup>+</sup> T cells exited the node there was a corresponding increase in the expression of the memory phenotype. Although the expression of class II coincided with the increase in activation/adhesion molecule expression, class II expression was upregulated on CD4<sup>+</sup> and CD8<sup>+</sup> T cells leaving the node for a longer period. These data suggest that



the increased expression of adhesion/activation markers correlates with the increased traffic of T cells through the node. Because class II expression on the T cell subsets was detectable for a longer period, its presence may be indicative of a different functional role.

Expression of the class II locus products on  $\gamma\delta$  T cells was observed on cells derived from afferent, efferent and peripheral blood. Similar to the results for CD4<sup>+</sup> and CD8<sup>+</sup> T cells, levels of expression were higher on afferent lymph than efferent lymph and peripheral blood  $\gamma\delta$  T cells, albeit at lower densities throughout. However, no discernible differences in levels of expression of the adhesion/activation markers were detected between afferent, efferent and peripheral blood. Assuming that CD45 expression correlates with activation status on  $\gamma\delta$  T cells as it does on  $\alpha\beta$  T cells, the lack of CD45R expression would indicate that the vast majority of  $\gamma\delta$  T cells are memory phenotype. The higher levels of  $\alpha 4$ -integrin expression are also indicative of a memory phenotype, but the high levels of L-selectin expression cannot be reconciled with that of a memory phenotype ( $\alpha 4$ -integrin<sup>hi</sup>, L-selectin<sup>lo</sup>). It was therefore not possible to correlate class II expression on  $\gamma\delta$  T cells with activation status.

In several species  $\gamma\delta$  T cells comprise a major, if not exclusive, T cell component of epithelial tissues (Allison and Havran,1991). Distribution of  $\gamma\delta$  T cells in mice show a strictly epidermal localization (Janeway *et al*,1988) while in sheep the intestinal epithelia and lamina propria are populated with large numbers of  $\gamma\delta$  T cells (Hein and Mackay,1991).  $\gamma\delta$  T cells are believed to be involved in the protection of these epithelial surfaces. In sheep, spleen and lymph node  $\gamma\delta$  T cells localize to the regions of cellular traffic. It appears that  $\gamma\delta$  T cells home to epithelial surfaces and return to the blood via the lymph, rather than the blood to lymph node route of  $\alpha\beta$  T cells (Mackay,1991). The unusual pattern of adhesion molecule expression observed on  $\gamma\delta$  T cells may serve a different circulatory route to  $\alpha\beta$  T cells. This may account for the differences in surface expression of adhesion molecules observed on  $\gamma\delta$  T cells as opposed to  $\alpha\beta$  T cells.

No changes in the proportions of  $\gamma\delta$  T cells exiting the node were observed during antigen challenge. This is in contrast to the work of Mackay *et al* (1992) which documented increased proportions of  $\gamma\delta$  T cells exiting the node after BCG challenge. The failure to detect a similar response in these experiments may be a reflection on the antigen used. A recent report, argues that  $\gamma\delta$  T cells have a system

for specific T cell recognition that is alternate to the MHC presented peptide complex of  $\alpha\beta$  T cells (Tanaka *et al*,1995). Thus an antigen such as ovalbumin would not be detected by the  $\gamma\delta$  T cells whereas small non-peptide antigens of mycobacterial infections are recognized. The interesting observation for  $\gamma\delta$  T cells was the increased levels of class II expression during the immune response. It would appear that factors present in the lymph node are responsible for the general upregulation of class II on T cell subsets during the secondary immune response.

The stimuli for the increased expression of class II occur during secondary immune responses only (Hopkins *et al*,1993). Cytokines produced by activated T cells are possible candidates for the induction of class II expression.  $\gamma$ IFN is a possible candidate as it has been shown to be released into efferent lymph from antigen activated lymph nodes only during secondary immune responses (Emery *et al*,1990).  $\gamma$ IFN induces the expression of the CIITA gene which is involved in the control of class II expression (Steimle *et al*,1994). As there is no evidence of CIITA gene binding directly to the promoters of class II, it has been suggested that  $\gamma$ IFN may act as a tissue specific coactivator. Another inducer that has been well studied is IL-4 which induces a DNA binding activity specific for the distal upstream region of a class II promotor (Gravallese *et al*,1991). However  $\gamma$ IFN and IL-4 alone are not responsible. When purified human T cells were activated, strong proliferation and IL-2 receptor expression was observed but low levels of class II expression. Addition of conditioned media resulted in increased surface class II expression but IL-2, IL-4 or  $\gamma$ IFN alone were responsible (Salvadori *et al*,1991). Work by Caplen *et al* (1992) has shown that resting T cells can transcribe mRNA for class II genes but that they do not express the protein product on the cell surface until after activation. In addition, they suggest that there may be a protein factor which may negatively influence class II levels in T cells.  $\gamma$ IFN may act as the tissue specific regulator of class II expression on T cells but other factors involved have yet to be identified. Promotor elements identified on the HLA-DRA, DPA and DQB genes show differential susceptibility to different cytokines (Kara and Glimcher,1993). Thus  $\gamma$ IFN may control the upregulation of class II on T cells while different cytokines may be responsible for the differential expression of the class II products.

Expression of MHC class II by APCs functions in the presentation of antigen to CD4 positive T cells. In addition, MHC class II delivers signals that modulate APC cell function. Similarly, class II molecules on human T cells have the ability to function as signal transduction elements. Crosslinking class II molecules induces



activation of tyrosine kinases and mobilization of intracellular calcium in activated human T cells (Odum *et al*,1991). Crosslinking class II with CD3 induces IL-2, IL-3,  $\gamma$ IFN and TNF $\alpha$  secretion by T cells (Spertini *et al*,1992). A more recent study has shown that crosslinking class II molecules on human T cells enhances the IL-2 proliferative response of DR<sup>+</sup> T cells (Odum *et al*,1993). This was elicited by an increase in IL-2 receptor expression on these T cells. It was found that the response was specific for DR expression, crosslinking DQ had no effect.

In the work presented here it was shown that Con A activation of purified DR<sup>+</sup> T cells induced IL-6 mRNA. The expression of IL-6 mRNA correlated with DR expression on T cells. IL-6 is a pleiotropic cytokine. One of IL-6's reported functions is its ability to replace the requirement for APC derived costimulatory signals for IL-2 secretion and proliferation (Kasahara *et al*,1990; Lorre *et al*,1990). Relating this to the work of Odum *et al* (1993), perhaps activation of DR<sup>+</sup> T cells induces IL-6 production which in turn induces IL-2 responsiveness. This may function to amplify a local immune response. Further studies are required to test this hypothesis.

Resting ovine T cell subsets express DR and little DQ. On activation DR expression predominates over DQ. Similarly in humans, DR expression predominates over DQ expression after activation. Crosslinking DR on human T cells has been shown to induce T cell sensitivity to IL-2 whereas crosslinking DQ had no effect (Odum *et al*,1993). The cytokine studies conducted in this thesis, correlated IL-6 mRNA production with T cell DR expression. This may indicate a functionally distinct immunoregulatory role for DR positive T cells. However, the expression of DQ was not analysed on these T cells after activation. From the work of Odum *et al* (1993) crosslinking DQ did not lead to IL-2 responsiveness. Perhaps, DQ expression on T cells may be associated with the induction of anergy that has been reported when using T cells as APCs (O'Hehir and Lamb,1990; LaSalle *et al*,1992). The differential expression of class II molecules may fulfill distinct functions in regulating the outcome of T cell activation and further experiments are required to delineate these functional roles.

Despite the increased expression of class II on T cells these cells do not induce antigen specific T cell proliferation (Hopkins *et al*,1993). However they do have the capacity to stimulate allogeneic T cells in an MLR (Hopkins *et al*,1993). Furthermore, it was shown that the ability to stimulate allogeneic T cells increases

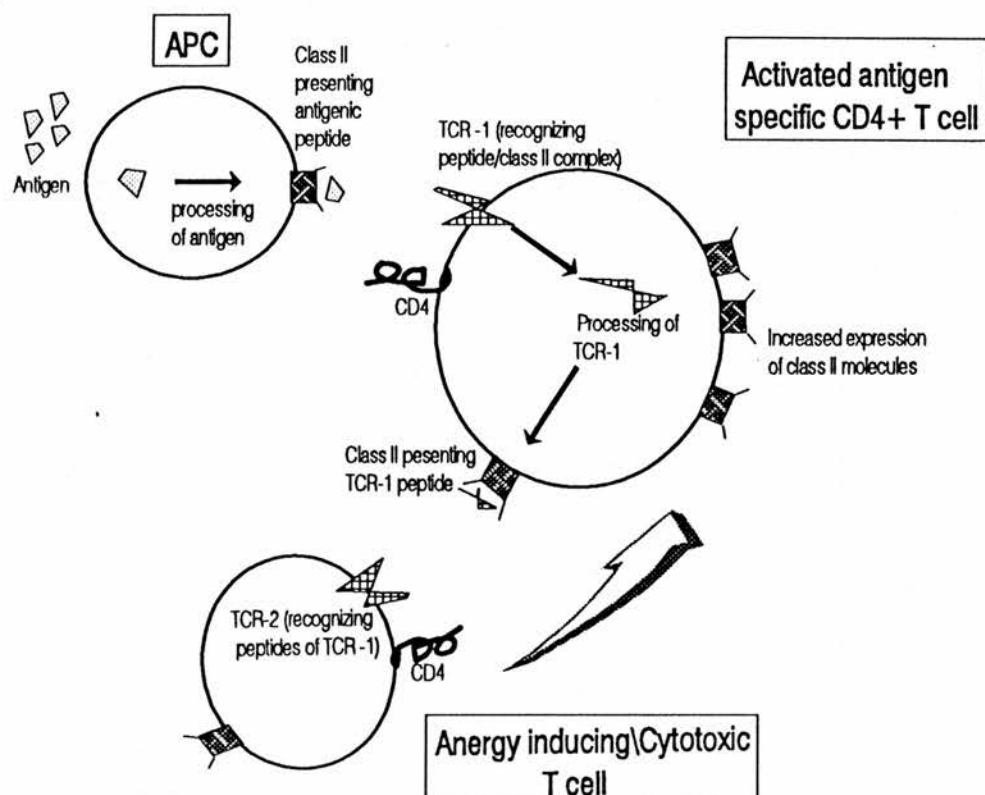
during *in vivo* secondary responses and correlates temporally and quantitatively with class II expression. The presentation of alloantigens by class II positive T cells may have significance in the immune response. Indeed many recent studies have implicated class II expression on T cells with the activation of autoreactive T cells. It is believed that activated T cells present peptides derived from activation associated molecules and peptides of the TCR. Broeren *et al* (1994) have shown that T cells can process and present their own TCR proteins to anti-TCR peptide-specific MHC class II restricted CD4<sup>+</sup> T cells. T cell regulation of activated T cells also underlies the principal of T cell vaccination. In patients with multiple sclerosis, there is an increased frequency of activated MBP reactive T cells (Allegretta *et al*, 1990). Vaccination of MS patients with irradiated autologous MBP reactive T cells induces an MHC restricted T cell response to the MBP reactive T cells (Zhang *et al*, 1993). The immunized patients developed cytotoxic CD8<sup>+</sup> and CD4<sup>+</sup> T cells that were reactive with MBP-specific clones and this decreased the frequency of MBP reactive T cells. It was postulated that this response was regulated by a clonotypic network whereby peptides of the TCR variable region were presented by T cells themselves. The upregulation of class II on T cells is coincident with the recently reported internalization/down-regulation of the TCR (Valitutti *et al*, 1995). Thus class II expression on T cells may regulate the immune response through T-T cell interactions (Figure 7.1A).

Class II positive human and rat T cells may regulate T-T cell interactions but whether this applies for sheep T cells remains to be established. Similar to the human and rat studies, sheep T cells also express class II on activation. However the expression of class II on sheep T cells differs from rat and human T cells in that a proportion of resting T cells are class II positive. The function of class II expression on resting T cells is unclear (Figure 7.1B). A T-T cell regulatory role for ovine T cells may not apply. A T-T cell regulatory role is also questionable in species that constitutively express class II on their T cells.

It would be interesting to conduct further studies to establish whether ovine class II positive T cells have an immunoregulatory role as has been suggested from rat and human studies. To establish a T-T cell regulatory role for class II positive T cells assays for autoreactivity (cytotoxicity assays) and anergy induction (proliferation assays) could be conducted. T cells derived from antigen challenged node could be tested in such assays using irradiated autologous antigen specific T cell clones as APCs. The role of class II positive resting T cells could also be assessed in

such assays. The analysis of peptides presented on class II positive T cells during an immune response may also delineate their functional role. From purified class II molecules, bound peptides may be identified by Edman microsequencing. It would then be interesting to compare the data on peptides analysed from class II positive activated T cells with that from resting T cells. Such experiments may elucidate the functional significance of class II expression on resting and activated ovine T cells. The analysis of peptides presented on DR and DQ molecules may delineate different functional roles for these molecules. The functional significance of class II positive T cells is a fascinating subject and further experiments are required to establish their precise role in the immune system.

A



B

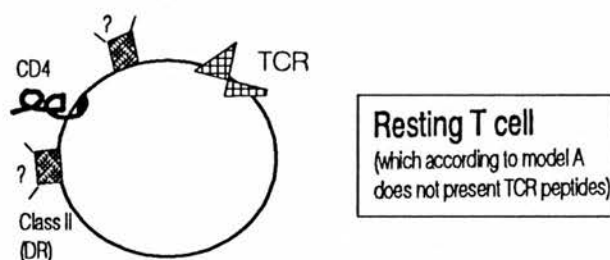


Figure 7.1 Schematic representation of class II function on ovine T cells.

(A) A model for the function of MHC class II expression on activated antigen specific T cells. The antigen specific T cell recognizes antigen presented on class II, this leads to the internalization of its antigen specific TCR and the increased expression of class II molecules. The TCR is processed in the class II pathway and presented to an anti-idiotypic T cell. Activation of the anti-idiotypic T cell may induce anergy or a cytotoxic response. Thus the immune response to antigen is downregulated.

(B) Resting T cells also express class II which according to model A do not present TCR peptides. The functional significance of class II expression on resting T cells has yet to be elucidated.

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## APPENDIX

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## Patterns of major histocompatibility complex class II expression on T cell subsets in different immunological compartments 1. Expression on resting T cells\*

In this study we have investigated the expression of major histocompatibility complex (MHC) class II molecules on T cells from various lymphoid compartments in the sheep. Monoclonal antibodies which react specifically with sheep MHC class II molecules homologous to the human DQ and DR molecules have been characterized. These antibodies have been used, together with the monoclonal antibodies specific for sheep CD4-, CD8- and T19-positive T cells, to quantitate DQ and DR expression on T cell subsets in adult and fetal peripheral blood, afferent lymph, lymph node and efferent lymph. The results show that expression of class II by T cells depends on the age of the animal and the physiological location of the T cell. In fetal blood there is no expression of class II on CD8<sup>+</sup> or T19<sup>+</sup> cells and very low expression on CD4<sup>+</sup> T cells. In adult peripheral blood and efferent lymph a significant proportion of cells express DR but not DQ. A very different situation is found in afferent lymph and the peripheral lymph node: in afferent lymph the majority of T cells in all three subsets express both DQ and DR molecules; in the lymph node over 50 % of T cells express DR and 30 % are DQ<sup>+</sup>. These results suggest that within all T cell subsets there is a progression from DQ<sup>-</sup> DR<sup>-</sup> to DQ<sup>-</sup> DR<sup>+</sup> and DQ<sup>+</sup> DR<sup>+</sup> which correlates with physiological stages of T cell differentiation *in vivo*.

### 1 Introduction

MHC class II expression by T lymphocytes shows considerable inter-species variation. In humans activated but not resting T cells are MHC class II-positive [1, 2] whereas in mice evidence for the expression of class II by T cells is controversial [3, 4]. All equine T cells constitutively express class II [5] and studies in sheep suggest that a proportion of T cells are class II-positive [6]. This is unlike the situation with dendritic cells and B cells which constitutively express class II in all species so far examined [7, 8]. The significance of class II expression on T cells is not clear. Class II-positive T cells can present peptide antigens and function as antigen presenting cells in an MLR [9–12] but their ability to present soluble antigens is limited by their lack of an efficient mechanism of antigen capture [13].

One approach to understanding the significance of T cell class II is to characterize its expression on T cell subsets isolated from different parts of the recirculating lymphocyte pool. This is only possible in sheep where lymphatic cannulation techniques permit an overall picture of physiological changes in lymphocyte phenotype and function to

be studied. We have chosen to examine phenotypic changes in class II on T cells because it is known that T cells drawn from different immunological compartments vary in their cell surface phenotype. Sheep afferent lymph T cells express memory cell surface phenotype while efferent lymph contains T cells of mainly naive phenotype and peripheral blood contains a mixture of the two phenotypes [14]. These phenotypic differences include differential expression of CD45 isoforms and quantitative variation of expression of LFA-1, LFA-3 and CD2. Whether these phenotypic differences identify fixed subsets of T cells or whether they reflect T cells at different stages of activation as a consequence of their physiological location is not known. While it is clear that CD45RO<sup>+</sup> cells have recall function *in vitro* [15, 16], the inflexibility of cell-surface phenotype has been called into question. In the rat, CD45RO<sup>+</sup> cells can revert to CD45RA<sup>+</sup> [17] and there is evidence that high levels of expression of LFA-1 can more properly be regarded as a marker of activation than as a memory cell marker [18].

In this paper we describe the development of a panel of monoclonal antibodies which recognize ovine DQ and DR homologues and use these antibodies to analyze expression of MHC class II molecules on T cell subsets isolated from different immunological compartments. The data show that in sheep there is considerable expression of MHC class II molecules on small resting T lymphocytes but that the products of the different loci are differentially expressed in all compartments. Furthermore, the class II phenotype of T cells varies significantly between different sites and is dependent on the age of the animal. These data indicate that expression of DR correlates with a differentiation state of T lymphocytes while DQ<sup>+</sup> cells appear to represent a population of recently activated cells. Our results show that T lymphocyte phenotype is not fixed but is continuously modulated during recirculation.

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## 2 Materials and methods

### 2.1 Anti-sheep monoclonal antibodies

Two panels of anti-sheep monoclonal antibodies were used in this study, one defining the major lymphocyte phenotypes and the other recognizing the sheep MHC class II locus products. These antibodies are listed in Table 1. The anti-sheep class II monoclonal antibodies have been described previously [6, 19]. These antibodies react with all sheep tested ( $> 30$ ) and appear to react with monomorphic determinants. VPM30 is a pan-sheep B cell monoclonal antibody which is not directed against Ig [20]. CC15 is an anti-bovine T19 monoclonal antibody which recognizes CD4<sup>+</sup>CD8<sup>+</sup> T cells expressing the gamma/delta T cell receptor [21]. This monoclonal antibody was a generous gift from Dr. C. Howard. SBUT4 and SBUT8 are specific for sheep CD4 and CD8 [22].

### 2.2 Preparation of cells

Sheep aged between 12 and 18 months were obtained from the Moredun Research Institute, Edinburgh. Prefemoral afferent lymph was collected by ablation of the prefemoral lymph node and subsequent cannulation of the efferent duct [23]. This duct now contains afferent lymph and is known as a "pseudoafferent". Efferent lymph cells were obtained by cannulation of the prefemoral efferent lymphatic [24]. Animals were allowed at least seven days post-operative recovery before lymph cells were taken for examination. Peripheral blood lymphocytes were isolated from heparinized blood by  $\text{NH}_4\text{Cl}$  lysis as described by Mishell and Shiigi [25]. Fetal blood was obtained from animals at 130 days gestation and lymphocytes were isolated as described above. Lymph node lymphocytes were obtained from prefemoral lymph nodes and splenocytes were prepared from spleens obtained from the local abattoir. Cells were teased into suspension and lymphocytes were obtained by centrifugation over Lymphoprep (Nycomed AS, Oslo, Norway).

### 2.3 Immunopurification and ELISA analysis of sheep class II

Immunoglobulin was prepared from anti-sheep class II monoclonal antibody ascites fluid by caprylic acid precipitation [26] and linked to Affigel 10 (Bio-Rad, Hemel Hempstead, GB). Sheep class II molecules were purified from spleen by affinity chromatography as described previously [6]. ELISA for detection of purified class II were carried out as described previously [6]. Both antibodies and antigens were titrated to give optimal concentrations.

### 2.4 Two-dimensional immunoblot analysis

For the analysis of  $\beta$  chains sheep afferent lymph cells were lysed at a concentration of  $8 \times 10^6/\text{ml}$  in 2% (w/v) NP40, 8.5 M urea containing 2% (v/v) 2D Pharmalyte (Sigma Chemical Company, Poole, Dorset, GB). Cells ( $0.4 \times 10^6 - 2 \times 10^6$ ) were fractionated by 2D NEPHGE-SDS (two-dimensional non-equilibrium pH gradient gel electrophoresis-SDS) polyacrylamide gel electrophoresis

**Table 1.** Monoclonal antibodies used

Antibody	Specificity	Reference
SW73.2	Anti-sheep MHC II pan $\beta$ chain	[19]
VPM36	Anti-sheep MHC II $\alpha$ chain	[6]
VPM38	Anti-sheep MHC II $\alpha$ chain	[6]
VPM41	Anti-sheep MHC II $\beta$ chain	[6]
VPM57	Anti-sheep MHC II $\beta$ chain	unpublished data
VPM30	Anti-sheep B cell	[20]
CC15	Anti-bovine T19	[21]
SBUT4	Anti-sheep CD4	[22]
SBUT8	Anti-sheep CD8	[22]

using a non-reduced gel in the second dimension [27]. For the analysis of  $\alpha$  chains sheep splenocytes were lysed at the same concentration in 2% (w/v) NP40, 8.5 M urea, 2% (v/v) pH 2.5–5 Pharmalyte (Sigma) and fractionated by 2D IEF-SDS polyacrylamide gel electrophoresis using a non-reduced gel in the second dimension [28]. The gels were blotted onto nitrocellulose, blocked with 5% (w/v) dried milk powder in PBS and incubated overnight in monoclonal antibody supernatant. Blots were incubated overnight in monoclonal antibody supernatant and developed by incubating in biotinylated anti-mouse Ig (Boehringer Mannheim, East Sussex, GB) or biotinylated anti-rat Ig (Sigma) followed by streptavidin alkaline phosphatase (Boehringer Mannheim) and nitroblue tetrazolium/bromochloroindole phosphate.

### 2.5 N-terminal sequence analysis of class II molecules

Affinity-purified class II molecules were separated into  $\alpha$  and  $\beta$  chains by SDS polyacrylamide gel electrophoresis using an Applied Biosystems HPEC 230A (Applied Biosystems, Warrington, Cheshire, GB). Separation was achieved on 10% polyacrylamide gels in 75 mM Tris phosphate pH 7.5 [29]. The separated eluted  $\alpha$  and  $\beta$  chains were centrifuged onto Problot membrane (Applied Biosystems) and sequenced by the SERC Protein Sequencing Facility, University of Aberdeen, GB, on an ABI pulsed liquid analyzer.

### 2.6 Flow cytometry

Immunoglobulin of the anti-cell phenotype antibodies, with the exception of VPM30, was isolated from ascitic fluid by caprylic acid treatment [26] followed by ammonium sulfate precipitation. VPM30 IgM was purified by S300 (Pharmacia, Milton Keynes, Bucks, GB) gel filtration chromatography. Purified Ig were then coupled to biotin (biotinamidocaproate N-hydroxysuccinamide ester) (Sigma) at a biotin/Ig ratio of 75  $\mu\text{g}/\text{mg}$ . Anti-class II monoclonal antibody Ig were treated similarly but were not coupled to biotin. The biotinylated anti-cell phenotype antibodies were detected using streptavidin-phycoerythrin (SA-PE, Amersham International PLC, Amersham, GB) and the anti-class II monoclonal antibodies were detected using the relevant Ig subclass-specific FITC-conjugated anti-mouse Ig antiserum (The Binding Site, Birmingham,



GB). Before use, the concentrations of all reagents were optimized by checkerboard titrations.

Cells ( $10^6$ ) of each cell population were stained simultaneously with 50  $\mu$ l of the appropriate dilution of biotinylated anti-phenotype monoclonal antibody and anti-class II monoclonal antibody diluted in PBS/0.1% BSA/0.01 M sodium azide (PBA). After 60-min incubation at 4°C the cells were washed twice in PBA and incubated for 60 min with SA-PE and FITC-antiglobulin before final washing and analysis. Cells ( $10^4$ ) live-gated on small lymphocytes were analyzed by flow cytometry using a Becton Dickinson FACScan Analyzer (Mountain View, CA). Live gating using forward scatter (FSC) and side scatter (SSC) parameters was used to distinguish the different cell populations in afferent and efferent lymph and blood [30]. Linear amplification was used for the physical parameters (FSC, SSC) and logarithmic amplification used for the fluorescence parameters (FL1-green, FL2-red). FL1-FL2 and FL2-FL1 compensation was used to optimize the two-color analysis. Antibody-negative cells were gated by reference to biotinylated normal mouse Ig at 10  $\mu$ g/ml followed by FITC anti-mouse IgG.

### 3 Results

#### 3.1 Monoclonal antibodies identify subgroups of sheep class II

The aim of these experiments was to identify which anti-sheep class II monoclonal antibodies reacted with different MHC class II molecules. The panel of monoclonal antibodies was characterized by immunopurifying the antigens reacting with each antibody and reacting the purified antigen with all other monoclonal antibodies by ELISA. Affinity purification of class II results in both  $\alpha$  and  $\beta$  chains being isolated regardless of the chain specificity of the monoclonal antibodies [19]. Table 2 shows the results of such an ELISA. OD readings are comparable except with VPM41 antigen which contains lower levels of antigen than the other preparations. These data show that there are two mutually exclusive groups of class II molecules: those defined by VPM36 and VPM41 (36/41) and those defined by VPM38 and VPM57 (38/57).

#### 3.2 Two-dimensional gel analysis of sheep class II

In order to further define the different subgroups of sheep class II, two-dimensional gel analysis of the  $\alpha$  and  $\beta$  chains

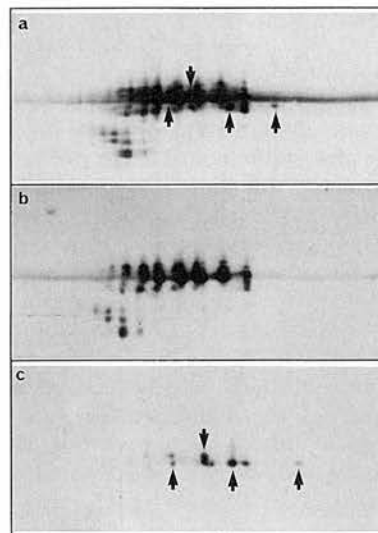


Figure 1. Two-dimensional immunoblots of sheep class II  $\beta$  chains. Sheep afferent lymph cell lysates were fractionated by NEPHGE 2D PAGE, transferred to nitrocellulose and developed with monoclonal antibodies. (a)SW73.2; (b)VPM57; (c)VPM41. Arrows indicate spots common to SW73.2 and VPM41 blots which are not present in VPM57 blots.

was carried out. Fig. 1 shows the sheep class II  $\beta$  chain spots. Fig. 1a shows the total  $\beta$  spots identified by the pan- $\beta$  chain rat monoclonal antibody SW73.2, Fig. 1b and c show identical blots probed with VPM57 (1b) or VPM41 (1c). Arrows in 1a and c indicate spots which are absent in Fig. 1b. Spots in Fig. 1c which are not arrowed are not resolved from VPM57 $\beta$  spots. The data shows that VPM57 and VPM41 identify different patterns of spots which are subgroups of total class II. No spots were observed with either mouse or rat negative control antibodies (data not shown). Fig. 2 shows the sheep class II  $\alpha$  chain spots identified by VPM36 (2a) and VPM38 (2b). These two antibodies clearly recognize different molecules.

#### 3.3 N-terminal amino acid analysis of sheep class II molecules

In order to determine the relationship between the sheep class II subgroups 36/41 and 38/57 and the subgroups of human and mouse class II, the N-terminal amino acid sequence of VPM36 and VPM38  $\alpha$  chains was determined. Fig. 3 shows the N-terminal amino acid sequence of

**Table 2.** Cross-reactivities of a panel of anti-sheep MHC class II monoclonal antibodies with the corresponding affinity purified class II molecules

Antibody <sup>b)</sup>	Chain Specificity	Antigen <sup>a)</sup>				
		SW73.2	VPM36	VPM41	VPM38	VPM57
SW73.2	$\beta$	1.076 <sup>c)</sup>	1.353	0.367	1.560	1.121
VPM36	$\alpha$	1.539	0.868	0.296	0.091	0.0
VPM41	$\beta$	1.712	0.983	0.351	0.076	0.0
VPM38	$\alpha$	1.558	0.117	0.053	0.889	0.703
VPM57	$\beta$	1.392	0.0	0.012	0.685	0.598

a) MHC class II molecules purified by affinity chromatography with each monoclonal antibody; appropriate dilutions of each preparation in PBS/azide were coated onto an ELISA plate overnight at 4°C.

b) Monoclonal antibody used in ELISA; antibodies were used as 1/40 dilutions of saturated supernatant.

c) OD492 nm with the negative control subtracted.



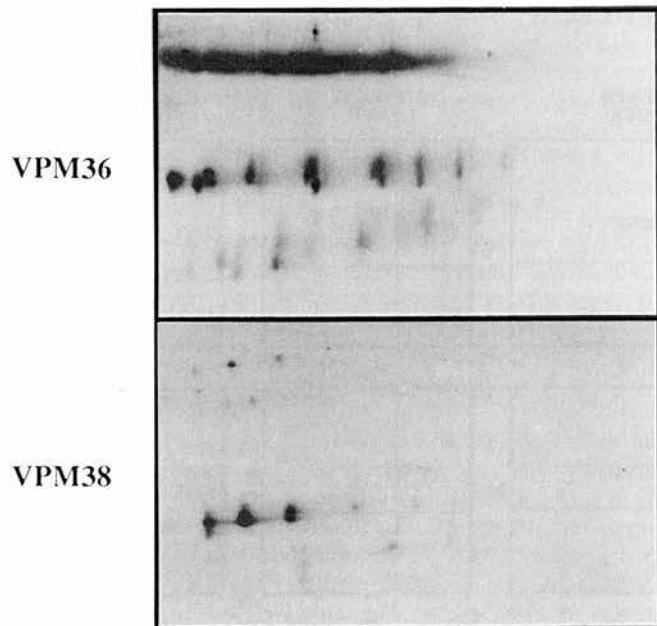


Figure 2. Two-dimensional immunoblots of sheep MHC class II  $\alpha$  chains. Sheep splenocyte lysates were fractionated by IEF 2D PAGE, transferred to nitrocellulose and developed with monoclonal antibodies. (a) VPM36; (b) VPM38.

VPM36  $\alpha$  and VPM38  $\alpha$  chains compared with human and mouse sequences [31]. The VPM36  $\alpha$  chain sequence is close to that of DQ $\alpha$  and I-A $\alpha$  and the  $\alpha$  chain sequence of VPM38 antigen is homologous to human DR $\alpha$  and mouse I-E $\alpha$ .

These data taken together with the ELISA and 2D blot data show that we have two mutually exclusive groups of monoclonal antibodies which define unambiguously the  $\alpha$  (VPM36) and  $\beta$  (VPM41) chains of the sheep DQ and the  $\alpha$  (VPM38) and  $\beta$  (VPM57) chains of the sheep DR (VPM38 and VPM57) molecules.

### 3.4 Expression of MHC class II *in vivo*

Previous experiments had shown that the anti-sheep pan-class II monoclonal antibody SW73.2 stained  $62 \pm 5\%$  of sheep resting PBMC [20]. This is higher than the percentage which can be accounted for by B cells. Two-color flow cytometry using SW73.2 and the anti-cell phenotype monoclonal antibodies showed that approximately 50% of the class II-positive cells are B cells. The remainder of class II-positive cells, which express class II at a lower and more variable level, are 27% CD4 $^{+}$ , 16% CD8 $^{+}$  and 13% T19 $^{+}$  (data not shown). This establishes that in the sheep class II is expressed on small resting T cells.

### 3.5 Differential expression of MHC class II by T cells

Having shown that peripheral blood resting T cells express class II we then used the anti-DQ (VPM36) and anti-DR (VPM38) monoclonal antibodies to investigate expression of the individual locus products *in vivo*. Class II expression on CD4 $^{+}$ , CD8 $^{+}$  and T19 $^{+}$  T cells in afferent

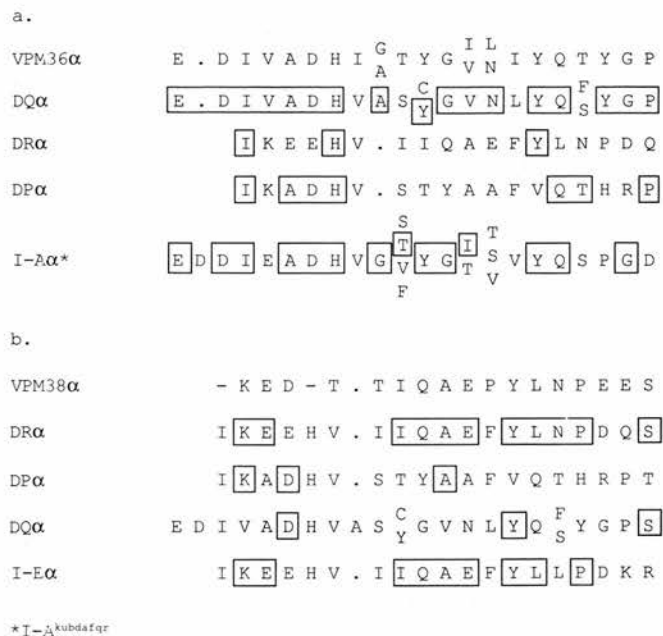


Figure 3. N-terminal amino acid analysis of (a) VPM36 $\alpha$  and (b) VPM38 $\alpha$  chains compared with human DP, DQ and DR and mouse I-A and I-E  $\alpha$  chain sequences. Boxes indicate identity with sheep sequence; "-" indicates that residues could not be determined; "." indicates a gap inserted to maximize alignment. Alternative residues indicate different alleles for human and mouse sequences. For sheep sequences alternative residues indicate that more than one residue was present. Human and mouse sequences taken from Travers and McDevitt [31].

and efferent lymph as well as peripheral blood and in the lymph node was investigated. To assess the effect of natural antigenic exposure on class II expression we examined class II subtype expression on fetal blood lymphocytes.

Fig. 4 shows the expression of DQ and DR molecules on CD4 $^{+}$  T cells drawn from the five different compartments. The class II positive population is not a discrete population, rather variable levels of class II appear to be expressed on individual T cells leading to the appearance of a continuum. These results are consistent over a large number of animals. The data show that CD4 $^{+}$  T cells from all compartments express DR molecules but the number of DR $^{+}$ CD4 $^{+}$  T cells varies between the different immunological environments. Approximately 90% of afferent lymph CD4 $^{+}$  T cells express DR but only 50% of lymph node and 30% of efferent lymph CD4 $^{+}$  T cells are DR $^{+}$  (Table 3). In fetal blood around 15% of CD4 $^{+}$  T cells express DR while in adult sheep (> 1 year of age) 60% of peripheral blood CD4 $^{+}$  T cells are DR $^{+}$ . Expression of DQ molecules on CD4 $^{+}$  T cells differs considerably from DR expression. In peripheral blood, fetal blood and efferent lymph very few CD4 $^{+}$  T cells express the DQ molecule. Significant expression of DQ is only found in afferent lymph and lymph node where 80% and 33% respectively of CD4 $^{+}$  T cells express DQ.

Expression of DQ and DR molecules on CD8 $^{+}$  T cells mirrors the expression on CD4 $^{+}$  T cells with the exception of fetal blood where there is no detectable expression of

CD4+ T CELLS

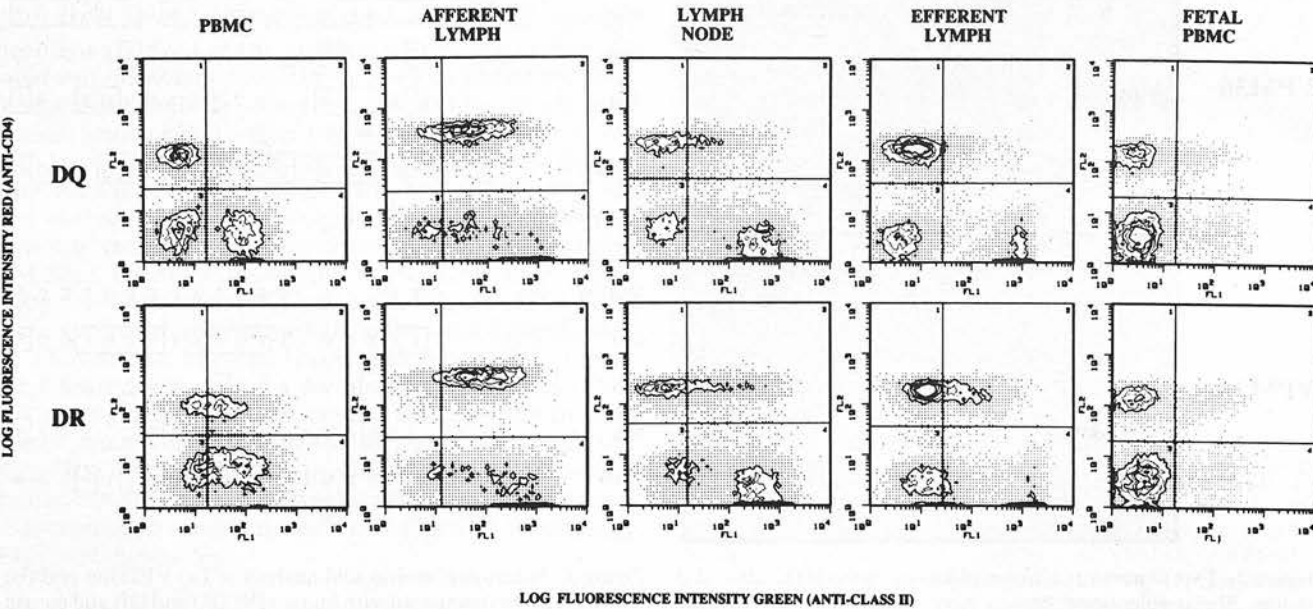


Figure 4. FACS analysis of lymphocytes from adult sheep peripheral blood (PBMC), afferent lymph, lymph node, efferent lymph and peripheral blood taken from fetuses at 130 days gestation (fetal PBMC) double-stained with the anti-CD4 monoclonal antibody SBU-T4 and the anti-class II subgroup-specific monoclonal antibodies VPM36 (anti-DQ) and VPM38 (anti-DR). SBU-T4 was coupled to biotin and detected with streptavidin-labeled phycoerythrin (red fluorescence) and the anti-class II monoclonal antibodies detected with isotype-specific FITC labeled anti-mouse Ig (green fluorescence).

either locus product on CD8<sup>+</sup> T cells (Table 3). In afferent lymph expression of both locus products is uniformly high. In adult peripheral blood very few CD8<sup>+</sup> T cells express DQ whereas a significant percentage express DR. The percentage of CD8<sup>+</sup>DR<sup>+</sup> T cells is, however, higher in blood than in efferent lymph. In the lymph node 30 % of CD8<sup>+</sup> T cells are DQ<sup>+</sup> and 50–60 % express DR molecules.

MHC class II expression on T19<sup>+</sup> T cells is generally lower than on CD4<sup>+</sup> and CD8<sup>+</sup> T cells but the pattern is similar (Table 3). DQ and DR are differentially expressed in all compartments. Very few T19<sup>+</sup> T cells in peripheral blood or efferent lymph are DQ<sup>+</sup> but in afferent lymph and the lymph node ≥ 50 % of cells are DQ<sup>+</sup>. Fetal T19<sup>+</sup> T cells, which comprise the majority of T cells in fetal blood, are DQ<sup>+</sup>DR<sup>+</sup>.

In contrast to the results with T cells there is no differential expression of class II molecules on B cells from any of the lymphoid compartments with all B cells expressing DQ and DR (data not shown).

4 Discussion

The aim of these studies was to develop and characterize monoclonal antibodies which are specific for the sheep homologues of DQ and DR, and to use these monoclonal antibodies to link T cell expression of MHC class II with the recirculation pathways of T cell subsets.

Using a combination of affinity purification and ELISA analysis we have described four monoclonal antibodies

Table 3. Percentage of T cells from different lymphoid compartments expressing MHC class II DQ and DR molecules<sup>a)</sup>

		Tissue compartment				
		PBMC	Afferent Lymph	Lymph Node	Efferent Lymph	Fetal PBMC
		(n = 5)	(n = 4)	(n = 4)	(n = 8)	(n = 3)
CD4 <sup>+</sup> T cells	DQ	10 3.5 ± 2.8	50 80.1 ± 3.6	33.6 ± 4.6	2 7 ± 2	11.4 ± 2.5
	DR	57.1 ± 8.3	89.1 ± 8.3	51.6 ± 5.2	30 ± 3	15.9 ± 2.5
CD8 <sup>+</sup> T cells	DQ	9 6.4 ± 3.9	5 73.8 ± 4.2	30.3 ± 3.2	13.0 ± 2	< 1
	DR	51.4 ± 7.3	79.8 ± 6.8	56.5 ± 6.3	38.4 ± 4	< 1
T19 <sup>+</sup> T cells	DQ	4 < 1	18 60.6 ± 6.2	48.3 ± 4.7	6 ± 3	< 1
	DR	35.3 ± 9.3	78.7 ± 4.8	62.3 ± 8.2	19 ± 2	< 1

a) Lymphocytes from each tissue were double-stained with biotinylated anti-CD4, anti-CD8 or anti-T19 monoclonal antibody and VPM36 (DQ specific) or VPM38 (DR specific) monoclonal antibody. T cells were detected using streptavidin-phycoerythrin and class II staining was detected using isotype-specific anti-mouse FITC. Cells were analyzed by FACSscan and gated to include only small resting lymphocytes.

which react with the  $\alpha$  or  $\beta$  chains of two distinct subgroups of sheep MHC class II molecules. By a combination of two-dimensional immunoblot analysis on separated  $\alpha$  and  $\beta$  chains, together with N-terminal sequence analysis of affinity-purified class II, we have shown that the monoclonal antibodies VPM 36 and VPM 41 recognize the ovine homologue of DQ, whereas the other monoclonal antibodies VPM 38 and VPM 57 recognize DR. The N-terminal amino acid analysis of the  $\alpha$  chains of the two subgroups shows that there is a high degree of homology between ovine  $\alpha$  chains and the corresponding  $\alpha$  chains in the human. These monoclonal antibodies can therefore be used for the unambiguous identification of the ovine equivalents of the human DQ and DR locus products.

We have established that there are both qualitative and quantitative differences in T cell subset expression of class II, depending on age and physiological location of the cells. Two-color flow cytometry using the monomorphic anti-sheep class II monoclonal, SW 73.2, and anti-cell phenotype monoclonals, shows that up to 50 % of T cells in blood express class II. These cells originate from normal healthy animals and are non-activated small lymphocytes. Although it is difficult to detect *de novo* synthesis of class II by these T cells, data in the accompanying paper [32] shows that T cells synthesize class II following *in vivo* activation. In contrast to the sheep, human T cells express class II only after recent activation [1, 2] and equine T cells are 100 % class II-positive, even in the resting state [5].

Using the DR- and DQ-specific monoclonals we have shown that fetal blood T cells do not express DQ or DR in either the CD8<sup>+</sup> or T19<sup>+</sup> T cell subsets, and show low levels of expression of both DR and DQ only in the CD4<sup>+</sup> T cell subset. By contrast, in adult sheep PBMC there is a significant level of expression of DR on all T cell subsets. Despite these elevated levels of DR on T cell subsets in the blood, the level of DQ expression is low. Similar results are found in efferent lymph where T cells are virtually DQ<sup>-</sup> but cells from all three subsets express DR albeit at a lower level than is found in blood. A different T cell class II phenotype is apparent when the T cell subsets are isolated either from afferent lymph or the lymph node. In these compartments the majority of the T cell subsets now express DQ as well as DR (DQ<sup>+</sup>DR<sup>+</sup>).

Phenotypic differences between afferent and efferent lymph and blood have been documented by Mackay et al. [14] who used a variety of non-MHC class II markers to show that afferent lymph contains cells mainly of the memory phenotype while efferent lymph and blood contain a mixture of memory and naive phenotypes. Based on numbers alone, the expression of DR on T cells correlates with a memory phenotype in all compartments. Our preliminary experiments using three-color FACS analysis have so far shown that CD45RA<sup>+</sup> cells (naive) are DQ<sup>-</sup>DR<sup>-</sup>. In humans, expression of DQ by T cells seems to be a result of recent activation [33] and the data in the accompanying paper [32] suggests that this also applies to sheep T cells. Afferent lymph T cells have an activation profile; they have a high forward and side scatter in FACS analysis, have a high rate of spontaneous DNA synthesis [23] and express elevated levels of IL-2R (unpublished observations). We conclude that the pattern of MHC class II expression on T cells reflects different levels of

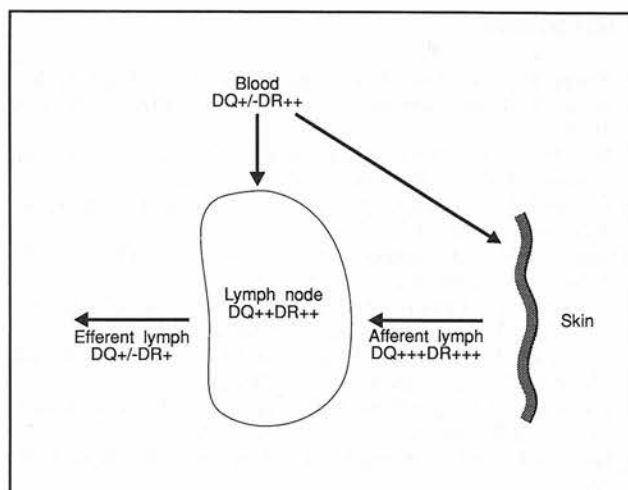


Figure 5. Expression of MHC class II molecules on T cells in different lymphoid compartments. +++: > 60 % positive; ++: 30–60 % positive; +: 20–30 % positive; ±: < 10 % positive.

T cell differentiation and activation. The DQ<sup>-</sup>DR<sup>-</sup> phenotype is typical of antigen-naïve fetal T cells. These change to being DQ<sup>-</sup>DR<sup>+</sup> and DQ<sup>+</sup>DR<sup>+</sup> after exposure to antigen. The DQ<sup>+</sup>DR<sup>+</sup> phenotype is characteristic of recently activated T cells present in afferent lymph and node and the DQ<sup>-</sup>DR<sup>+</sup> T cells represent a resting and recirculating memory T cell phenotype present in efferent lymph and blood (Fig. 5).

These data also establish that there is considerable plasticity of T cell phenotype. The migration of lymphocytes across endothelia into tissues (e.g. skin or lymph node) is a multistep process requiring adhesion, activation of  $\beta_2$  integrins on the lymphocyte surface and trans-endothelial migration [34, 35]. Crucially this process requires activation which occurs via endothelium/T cell interaction or endothelial-derived factors. High levels of DQ and DR in afferent lymph may therefore be a consequence of this activation. The explanation for the low level of DQ expression in efferent lymph may reflect the fact these cells do not appear in efferent lymph until about 30 h after they have crossed the endothelium [36] and by this time they have reverted to a DQ<sup>-</sup>DR<sup>+</sup> phenotype. In rats, T cells can revert their phenotype from CD45RO<sup>+</sup> to CD45RA<sup>+</sup> [17] and we propose that the reversion from DQ<sup>+</sup>DR<sup>+</sup> to DQ<sup>-</sup>DR<sup>+</sup> is another example of modulation of T cell phenotype which occurs at different stages in the recirculation pathway. The functional significance of differential expression of MHC class II on T cells is discussed in the accompanying paper [32].

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